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J.A. Hunter
(Ph.D. Thesis)

March 1983

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THE INTERACTION OF PLASMA LIPOPROTEINS WITH PHOSPHATIDYLCHOLINE VESICLES

James Arthur Hunter
(Ph.D. Thesis)

Biology and Medicine Division
Lawrence Berkeley Laboratory
University of California
Berkeley, Ca 94720

March 1983

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The Interaction of Plasma Lipoproteins
with Phosphatidylcholine Vesicles

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By

James Arthur Hunter
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ABSTRACT

In these studies, plasma lipoproteins were incubated with small unilamellar vesicles of phosphatidylcholine, and the interaction products were characterized in terms of chemical composition and physical-chemical properties.

Separate interactions (37 °C) of all density subclasses of high density lipoproteins (HDL) with vesicles resulted in the formation of three products: liposomes with associated apolipoproteins, discoidal complexes of phospholipids and apolipoproteins, and lipoproteins with an altered chemical composition. The lipoprotein product resulted from phospholipid uptake by and apoprotein dissociation from the density subclasses of HDL (HDL₃, HDL₂a, and HDL₂b). The increase in phospholipid content of these subclasses was highly correlated with the decrease in apoprotein content.

The major protein component of HDL, apolipoproteinA-I (apoA-I), was the primary apolipoprotein dissociated from the subclasses. Dissociation of apolipoprotein had not reached a limiting value for any subclass in the range of molar ratios (PC:HDL) investigated. At the highest molar
ratios for the incubation mixtures, the average number of apoA-I molecules dissociated per HDL particle was 0.75:1, 1.2:1, and 2.1:1 for HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b}, respectively.

Phospholipid uptake by HDL\textsubscript{3} and HDL\textsubscript{2a} subclasses did not reach saturation levels at the highest molar ratios investigated; the maximum uptake observed for these density subclasses was 36:1 and 68:1 (PL:HDL), respectively. Uptake by HDL\textsubscript{2b} reached a limiting value of 48:1 (PL:HDL).

For all HDL subclasses, the discoidal product had a mean hydrated density in the range of 1.051-1.056 g/ml. The average phospholipid to protein weight ratio for this product, isolated from separate mixtures containing HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b}, was 4.98:1, 6.52:1, and 4.06:1, respectively. The predominant protein component of this product was apoA-I; minor amounts of apoA-II were also observed. The discoidal product accounted for greater than 75\% of the dissociated apolipoprotein.

For all HDL subclasses, the liposomal product was isolated from incubation mixtures at high molar ratios (PC:HDL). The average phospholipid to protein weight ratio for this product was in the range of 20:1 to 22:1 for HDL\textsubscript{3} and HDL\textsubscript{2a} but ranged from 5:1 to 12:1 for HDL\textsubscript{2b}. For HDL\textsubscript{3} and HDL\textsubscript{2a}, apoA-I was the only electrophoretically-detected protein component; for HDL\textsubscript{2b}, A-I and minor amounts of the C apolipoproteins
were demonstrated.

Between 60 and 75% of the phospholipid added to incubation mixtures was generally accounted for by the discoidal product. Approximately 20 to 30% of the "added" phospholipid was accounted for by the lipoprotein product; the remainder was isolated as the liposomal product (less than 30%).

Phospholipid uptake by and apolipoprotein dissociation from subclasses of HDL resulted in a progressive decrease in mean hydrated density and a gradual increase in mean diameter for these lipoproteins.

For interaction mixtures at molar ratios of 150:1 and 295:1 (PC:HDL), the mean diameter of the (HDL\(_{3a}\))\(_{gge}\) size subpopulation entered the size range of the (HDL\(_{2a}\))\(_{gge}\) subpopulation after a 6 h incubation. At molar ratios above 40:1 for (HDL\(_{3a}\))\(_{gge}\) and 150:1 for (HDL\(_{3b}\))\(_{gge}\), their mean diameters shifted into the (HDL\(_{2a}\))\(_{gge}\) diameter range after a 24 h incubation.

The mean diameters of both (HDL\(_{2a}\))\(_{gge}\) and (HDL\(_{2b}\))\(_{gge}\) subpopulations remained within their respective size ranges, after a 6 h incubation, at all molar ratios investigated. After 24 h, however, the mean diameter of the (HDL\(_{2a}\))\(_{gge}\) subpopulation increased to values within the range of the (HDL\(_{2b}\))\(_{gge}\) subpopulation for molar ratios above 345:1 (PC:HDL); the mean diameter of the (HDL\(_{2b}\))\(_{gge}\) subpopulation remained within the size range of the normal (HDL\(_{2b}\))\(_{gge}\) subpopulation.
Interaction of lipoproteins containing apolipoprotein B with vesicles resulted in lipoprotein-vesicle aggregation. For interactions of low density lipoproteins (LDL) and vesicles, two products were described: lipoproteins with an altered chemical composition and LDL-vesicle aggregates. The LDL-vesicle reaction kinetics, monitored spectrophotometrically, suggested that the aggregate initially has a 1:1 (LDL:SUV) molar stoichiometry. An Arrhenius plot of the initial reaction rates at temperatures of 5, 14, 24, 30, and 37 °C was non-linear; consequently, an activation energy was not determined. A plot of initial reaction rates versus temperature exhibited a minimum, which fell within a temperature range of 21-28 °C for different LDL samples.

After interaction periods of 24 h, phospholipid uptake by non-aggregated LDL was temperature dependent; at 5, 14, 30, and 37 °C, the increase in phospholipid content was 0%, 4%, 23%, and 31%, respectively. Uptake of phospholipid was not associated with an increase in LDL diameter, determined by gradient gel electrophoresis.

The LDL-vesicle aggregation products, isolated from the mixtures that were incubated at 5, 14, 30, and 37 °C, had respective weight ratios of 2.83:1, 3.43:1, 3.98:1, and 3.46:1 (phospholipid:protein), which were 3-4 times that for native LDL (1.00:1). Two models for phospholipid uptake by LDL in the aggregation product were used to obtain the final molar stoichiometries of the LDL-vesicle
aggregates.

Aggregation of LDL with vesicles was less in mixtures containing both HDL and LDL than in those containing only LDL. For mixtures of total plasma lipoproteins, the extent of LDL-vesicle aggregation was inversely related to their respective HDL:LDL ratios. Thus, HDL can compete with LDL for vesicles.
To my Mother and Father....
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Epidemiological studies have demonstrated statistically significant correlations between the prevalence of cardiovascular disease and plasma levels of low density (LDL) and high density lipoproteins (HDL). The plasma concentration of LDL is positively correlated with the incidence of coronary heart disease, and the concentration of HDL is negatively correlated with this disease (57-63).

In order to understand the putative roles of LDL and HDL in atherogenesis, it is important to characterize their participation in lipid metabolism and to delineate the influence of metabolism on their physical-chemical properties. During active lipid transport in the bloodstream, degradation of triacylglycerol-rich chylomicrons (CM) and very low density lipoproteins (VLDL) markedly affects the chemical composition and physical-chemical properties of HDL in vivo (191,225); effects on LDL have not been studied. A major feature of the catabolism of CM and VLDL is the formation of phospholipid vesicles and of discoidal complexes of apolipoprotein and phospholipid (48,50,51,187,189). Vesicles of phosphatidylcholine can be produced in vitro and can be used in model studies with lipoproteins to characterize, under controlled conditions, processes occurring in vivo that may have relevance to
lipid metabolism and atherogenesis.

In this dissertation, I have investigated the in vitro interaction of small unilamellar vesicles with plasma lipoproteins, particularly HDL and LDL. Changes in lipoprotein chemical composition, diameter, and density were characterized during lipoprotein-vesicle interactions. Additional products formed during vesicle interaction with either HDL or LDL were isolated and characterized. These in vitro model studies provide additional insight into the role of vesicles in lipoprotein interconversions occurring in vivo and into the interaction products that may influence the atherogenic process. In addition, these findings contribute insights into the molecular details of lipoprotein structure.
I. INTRODUCTION

A. LIPOPROTEIN STRUCTURE

Plasma lipoproteins are spherical macromolecular complexes of lipids and proteins (apolipoproteins). For brevity, this introduction to the structure and function of lipoproteins will be limited to information particularly relevant for the findings to be described. Therefore, the interested reader is referred to a number of recent lipoprotein reviews for additional discussion (1-9).

Plasma lipoproteins, according to their hydrated densities, constitute 4 major classes: chylomicrons (CM) and very low density lipoproteins (VLDL) exhibit densities less than 1.006 g/ml, low density lipoproteins (LDL) occur within the 1.019-1.063 g/ml interval, and high density lipoproteins (HDL) are defined by densities within the 1.063-1.210 g/ml interval. Intermediate density lipoproteins (IDL) and lipoprotein (a) are minor lipoprotein classes that have densities within the 1.006-1.019 g/ml and 1.050-1.120 g/ml ranges, respectively.

All prevailing models describing the general organization of lipoprotein components propose a core of apolar lipids, cholesteryl esters and triacylglycerols, surrounded by a monolayer of phospholipids containing
<table>
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<th>CM</th>
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Major Apolipoproteins

- B-100
- B-48
- C-I
- C-II
- C-III
- E

Minor Apolipoproteins

- A-I
- A-II
- D

Abbreviations for lipoprotein classes are described in the text.

*Protein and lipid moieties are reported as a percentage of the total lipoprotein molecular weight.

**Specific lipid classes are reported as a percentage of the total lipid mass.

Data compiled from references 1-9, 239, 281.
apolipoprotein(s) and free cholesterol (10-13). These models differ, however, in the detailed molecular organization and interaction of the constituents.

B. LIPOPROTEIN CHEMICAL COMPOSITION

The organization of lipoprotein components into a surface monolayer and a central core allows for considerable variation in lipid and apolipoprotein composition among the lipoprotein classes (Table I). The predominant lipid and apolipoprotein components transported by the lipoproteins are normally quite specific for a particular class.

For CM and VLDL, triacylglycerols are the principal lipids and account for 85-90% and 37-57% of the total lipid mass, respectively (Table I). Total cholesterol (both unesterified and esterified) is the predominant lipid for LDL and accounts for 40-59% of the LDL lipid mass; the cholesteryl ester mass per particle is about 3 times that of unesterified cholesterol. Finally, the principal lipid carried by HDL is phospholipid, which accounts for 43-50% of the total lipid mass (14).

There are 8 major apolipoproteins distributed among the lipoprotein classes (Table I). They have been designated apolipoproteins A-I (apoA-I), A-II (apoA-II), B (apoB), C-I (apoC-I), C-II (apoC-II), C-III (apoC-III), D (apoD), and E (apoE) (15). For apoE, apoB, and apoC-III,
subspecies have also been described (239,282,283). Apolipoprotein F (apoF) is a minor apolipoprotein component which is isolated within the HDL and LDL density regions (281). Table I shows that CM contain the A-I, A-II, B, C-I, C-II, and C-III apolipoproteins. Very low density lipoproteins contain apolipoproteins B, C-I, C-II, C-III, and E as the major protein components and trace amounts of apoA-I and apoA-II. Low density lipoproteins contain apoB as the predominant protein component. Except for apoB, HDL contain all of the major apolipoprotein components; apoA-I and apoA-II are the major protein constituents for this lipoprotein class (1-9).

In addition to differences in apolipoprotein composition among the major lipoprotein classes, several studies indicate that apolipoproteins are not distributed uniformly within these classes. Thus, early studies on VLDL suggested differences in the apolipoprotein distribution among these large lipoprotein particles (16). More recent evidence shows that there is a progressive decrease in the content of the C and E apolipoproteins with decreasing particle size of the VLDL (17). The distribution of apolipoproteins in association with HDL is also not uniformly distributed among the lipoprotein particles constituting this class (18-20). Thus, particles are found that contain predominantly either A or C apolipoproteins (19,20). However, the C apolipoproteins account for less than 25% of the protein of the total HDL
class. Particles with a 2:1 molar ratio of apoA-I to apoA-II are observed throughout the HDL density range (21,22). Particles containing only apoA-I appear in the 1.063-1.075 g/ml density region of the HDL distribution (22). The C and E apolipoproteins are also associated with HDL particles in this density range (21).

C. LIPOPROTEIN PHYSICAL-CHEMICAL PROPERTIES

Lipoprotein physical-chemical properties are affected by the composition of the different lipoprotein species. The relative contents of the apolipoprotein and lipid moieties determine the hydrated densities of the major lipoprotein classes. Lipids typically exhibit densities less than about 1.03 g/ml, while proteins have densities in the range of 1.30-1.35 g/ml. The hydrated densities of CM (about 98% lipid) and of VLDL (about 90% lipid) are less than 1.006 g/ml; LDL (about 80% lipid) and HDL (about 55% lipid) have densities in the range of 1.019-1.063 g/ml and 1.063-1.210 g/ml, respectively (Table I).

Differences in the hydrated densities are exploited for ultracentrifugal isolation of lipoprotein classes in preparative amounts. Isolation of the major lipoprotein classes is ordinarily performed by sequential ultracentrifugation at the appropriate background solution densities (23).

In addition to variations in hydrated density,
differences in the chemical composition of the major lipoprotein classes are also associated with differences in physical-chemical properties such as diameter, molecular weight, and ultracentrifugal flotation rate (1-9). (The ultracentrifugal flotation rate is defined as the negative of the ultracentrifugal sedimentation rate.)

Besides a heterogeneity of parameters among the major lipoprotein classes, heterogeneity in chemical composition and physical-chemical properties is also observed within each of the major lipoprotein classes (24-34). The equilibrium distribution of LDL particles throughout the density interval of 1.0268-1.0597 g/ml has been investigated and found to vary among individuals (25). For isolated density subfractions of LDL, changes in chemical composition have been associated with variations in mean hydrated density. The frequency distribution of mean particle diameters of size subpopulations in LDL has been examined by gradient gel electrophoresis (GGE). Although there is individual variation in the number of subpopulations detected, the frequency distribution of mean particle sizes shows several well defined modes (29). Despite the heterogeneity of physical-chemical properties observed for LDL, relationships among these properties have been observed. There is an inverse relationship between hydrated particle density and both diameter and ultracentrifugal flotation rate. A direct relationship has been obtained for particle diameter and flotation rate.
High density lipoproteins also exhibit heterogeneity in chemical composition and physical-chemical parameters. From the work of Anderson et al., three subclasses of HDL are isolated by equilibrium density gradient ultracentrifugation (24). The subclasses are designated HDL3, HDL2a, and HDL2b; they band during ultracentrifugation within density intervals of 1.125-1.210 g/ml, 1.100-1.125 g/ml, and 1.063-1.100 g/ml, respectively. Differences in chemical composition are associated with variations in the mean hydrated densities of the HDL subclasses (24). These HDL species demonstrate an inverse relationship between subfraction density and particle molecular weight.

Recently, the frequency distribution of HDL particle sizes has been examined by GGE in a human population sample and found to be characterized by 5 modes (34). The diameter intervals containing the modes are used to characterize five HDL size subpopulations; not all subjects, however, have the five subpopulations.

There is a one-to-one correspondence between the ultracentrifugal density subclasses of HDL2b or HDL2a and specific subpopulations, identified electrophoretically. These size subpopulations are designated (HDL2b)gge and (HDL2a)gge, respectively. The HDL3 density subclass comprises one or more of three size subpopulations, (HDL3a)gge, (HDL3b)gge, and
(HDL₃C)gge.

A variety of other techniques have also been employed to fractionate HDL subpopulations. Hydroxyapatite column chromatography of total HDL gives 11 apparent subpopulations (35). Ion exchange chromatography, employing diethylaminoethane-cellulose and a linear salt gradient, yields three major subfractions of HDL (36). The HDL are fractionated by heparin-sepharose affinity chromatography into multiple species (37-39). Typically, there is a subfraction that contains little or no apoE but has the A and C apolipoproteins; this subfraction is not retained by the column matrix. The subfraction that is retained has apoE in addition to the A and C apolipoproteins. All of the chromatographic techniques described above yield multiple fractions within the ultracentrifugally designated HDL density subclasses, HDL₂ (1.063-1.125 g/ml) and HDL₃ (1.125-1.210 g/ml). Rate zonal ultracentrifugation yields 3 HDL subfractions (40). One has a mean flotation rate, molecular weight, and hydrated density similar to that of HDL₂b, and "HDL₃L" has properties similar to those reported for HDL₃ (24). Finally, electrophoretic methods, other than GGE, have been used to characterize HDL (41-44). Electrophoresis of HDL₃ on 7.5% polyacrylamide gels demonstrates 3 subpopulations; on 3.75% gels, 5 subpopulations are observed (41). Isoelectric focusing indicates 12 components within HDL₃ in the pH range of 3.8 to 7.4, and
multiple components within HDL₂ (42-44).

The studies described above demonstrate that LDL and HDL are heterogeneous with respect to both composition and physical-chemical properties. This heterogeneity can be utilized to fractionate the lipoprotein classes into a number of subpopulations. In many cases, the fractionation procedures rely on a combination of compositional and physical-chemical properties.

D. INFLUENCE OF LIPOPROTEIN METABOLISM ON PHYSICAL-CHEMICAL PROPERTIES

Density subfractions within a lipoprotein class which have distinct chemical compositions, have distinct physical-chemical properties. Thus, metabolic processes which alter either the absolute amounts of lipoprotein components or the relative amounts of protein to lipid will produce changes in one or more of the following physical-chemical parameters: diameter, flotation rate, molecular weight, or hydrated density. Indeed, both in vivo and in vitro metabolic studies of lipolytic degradation of the triacylglycerol component of CM or VLDL have described a reduction in diameter (46,47,49-51,67,186), flotation rate (45,50,51,182), and molecular weight (50,182,186), and an increase in density (48,50,57) for the product particles.

For HDL and LDL, relationships between compositional
changes of the lipoprotein, resulting from metabolism, and the associated changes in physical-chemical properties have not been extensively investigated. Recently, changes in HDL chemical composition have been described following interaction with lecithin:cholesterol acyltransferase (LCAT, E.C. 2.3.1.43). (This enzyme catalyzes the esterification of cholesterol with the acyl chain from the 2 position of phosphatidylcholine.) Associated with LCAT action, there is a decrease in hydrated density for HDL and an increase in flotation rate (151). An increase in particle diameter has also been noted for HDL after LCAT activity, but the compositional changes have not been determined (152).

A number of investigators have altered the chemical composition of HDL by non-enzymatic means and examined the physical-chemical properties of the resulting products (52-56). Dissociation of apolipoproteins by guanidine hydrochloride yields HDL particles with an increased diameter and flotation rate (52,53). During incubation of HDL with phosphatidylcholine vesicles, a decrease in apolipoprotein and an increase in phospholipid content of the lipoprotein results in an increase in mean particle size, a decrease in density (54-56), and an increase in flotation rate (56).

Thus, the heterogeneity of physical-chemical properties for lipoprotein classes and subclasses can reflect compositional changes, resulting from a variety of
steps in lipoprotein metabolism. In addition, it is conceivable that the synthesis and plasma removal of specific lipoprotein species also contribute to the observed in vivo lipoprotein polydispersity. The association between physical-chemical properties and chemical composition of lipoprotein subfractions is an important aspect of this dissertation. My studies have been directed, in major part, towards characterization of the changes in several physical-chemical parameters after in vitro modification of the lipoprotein composition and towards an understanding of the relationships between lipoprotein structure and metabolism.

E. LIPOPROTEINS AND AHEROGENESIS

LDL transport between 66 and 75% of the total cholesterol in the plasma of normal individuals; approximately 20 to 25% of plasma cholesterol is transported by HDL (61). Because atherosclerosis is characterized by abnormal cholesterol deposition, plasma lipoproteins that are rich in cholesterol have been implicated as pathogenic agents in this arterial disease. Indeed, epidemiologic studies have shown that the plasma concentration of LDL is directly correlated with the prevalence of coronary heart disease (59,61). On the other hand, the plasma concentration of HDL is inversely correlated with the prevalence of coronary heart disease.
Processes in the metabolism of LDL or HDL, which could affect their physical-chemical properties, may contribute to the putative roles of these lipoproteins in atherogenesis and are currently under intensive investigation. Detailed characterization of metabolically related changes in lipoprotein properties may provide important information for understanding factors contributing to atherosclerosis and for diagnosing individuals who have a high risk of developing cardiovascular disease.

F. METABOLISM OF LDL

The LDL are formed primarily during the metabolic degradation of VLDL by lipolytic enzymes located on the arterial surface (64,65). Chylomicrons only contribute a small fraction of the total plasma LDL (66). Once formed, LDL exhibit a half-life in plasma of about 3 days (68-72). There is conflicting evidence on the primary tissues and organs involved in removing LDL from the circulation. For rats, the liver and adrenal glands have been shown to be important organs for LDL removal (73,74). For pigs, a total hepatectomy did not result in a decreased catabolic rate of LDL; in fact, the removal of LDL from plasma was reported to increase (76). In contrast, studies of pigs, using LDL conjugated to $^{14}$C-sucrose, indicated that about 40% of the degraded LDL appeared in the liver. Although
the liver accounted for the largest percentage of catabolized LDL per organ, the adrenal glands were about 2.5 to 5 times as effective in terms of LDL binding per gm of wet tissue (75). For rabbits, the liver is the major site of LDL catabolism (100).

In addition to information concerning tissues and organs contributing to LDL removal, there are a number of studies on the interaction of LDL with high-affinity membrane receptors in a variety of cell types: endothelial cells (83,84), smooth muscle cells (77-79), hepatocytes (85), adrenal cells (82), macrophages (80,81), lymphocytes (89), and fibroblasts (86-88). Several review articles on this subject have appeared recently (90-93). The receptors on fibroblasts for LDL recognize both apolipoproteins B and E (94); the apoE binding affinity is greater than that for apoB (95,96). Scatchard analysis indicates that the receptor has multiple binding sites (97). Radiation inactivation experiments have suggested a molecular weight of approximately 100,000 for the receptor (97). Recently, the LDL receptor from adrenal cortex membranes has been purified and found to be an acidic glycoprotein with a molecular weight of 164,000 (98).

Besides high-affinity binding, cells also exhibit low-affinity binding of LDL with subsequent uptake and degradation (87). While a great deal of work has focused on the nature of the high-affinity receptor, very little information is available on the low-affinity pathway for
lipoprotein interaction with cells. Low-affinity binding has been shown to be independent of the high-affinity LDL receptors. It was proposed that bulk fluid endocytosis accounted for the low-affinity uptake of LDL by fibroblasts (87). Recent studies, however, of monocyte-derived macrophages have indicated that LDL uptake at concentrations of lipoprotein that saturate the high-affinity receptors could not be accounted for by fluid endocytosis (275).

The in vivo role of high-affinity versus low-affinity uptake of LDL has been investigated for several animal species. For rats, 67.4% and 69.5% of LDL removal by the liver and the adrenal glands is mediated by high-affinity receptors, respectively (99). For rabbits, high-affinity uptake accounts for 63% and 92% of the degradation by the liver and the adrenal glands, respectively (100).

During the plasma lifetime of LDL, they are involved in a number of metabolic processes. The LDL participate in the exchange of phospholipid and unesterified cholesterol with cells and with other lipoproteins (8,104-113). In addition, transfer and exchange of apolar lipids (cholesteryl esters and triacylglycerols) among lipoproteins have been demonstrated in the presence of plasma proteins (8,105,112-126); The identity of the protein component(s) involved is still in dispute. Some investigators propose that apoD functions as the cholesteryl ester exchange protein (116) while others
contend that a different plasma protein is involved (124).

G. METABOLISM OF HDL

In the rat, precursor species of HDL are synthesized both by the liver and by the intestine as discoidal-shaped particles (127-129). In vitro studies have demonstrated the conversion of these structures to spherical particles by the action of LCAT (130). Recently, hepatocytes isolated from hypercholesterolemic rats have been shown to secrete discoidal HDL (131). The possibility that human precursor HDL species are also discoidal particles has been suggested by studies on patients with an inborn deficiency of LCAT and on subjects experiencing LCAT deficiency secondary to alcoholic hepatitis (132,133). These subjects showed discoidal HDL species which could be converted to spherical normal-appearing HDL.

Data on the removal of HDL from the circulation are consistent with a two component model with a half-life in plasma ranging from 2.95 to 5.8 days for the slower (terminal) decay component (134-136). Recently, a residence time (1/fractional catabolic rate) of 5.07 and 5.96 days has been reported for apoA-I and apoA-II, respectively (137).

There are conflicting data concerning the primary organ sites for HDL uptake. In rats, the liver and small intestine are the organs primarily involved in HDL removal.
from plasma (138-142); the adrenal glands have the highest uptake of HDL per gram of tissue (139). Recently, rat liver perfusion studies, designed to quantitate the contribution of this organ to the plasma removal of HDL, have suggested that only about 7% of the in vivo removal could be accounted for by the liver (143). In dogs, the liver and kidneys are the primary sites for HDL uptake (144).

Studies on the interaction of HDL with cells isolated from the liver and the adrenal glands have demonstrated membrane binding sites that were saturable (139,145). The rate-limiting cellular enzyme for cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase, has been shown to be stimulated in rat hepatocytes by HDL (146).

The HDL are involved in a number of metabolic processes during their in vivo lifetime. They are the primary site for LCAT activity because of the cofactor role of apoA-I in this enzyme's function (147,148). The LCAT reaction has been extensively investigated and is the subject of several reviews (149,150,225). This enzyme catalyzes the transfer of the fatty acyl group from the 2 position of phosphatidylcholine to the 3-hydroxy position of cholesterol, which results in the formation of lysolecithin and cholesteryl ester (160,161). The LCAT enzyme has a molecular weight of 59,000 determined by sedimentation equilibrium measurements and of about 66,000 determined by sodium dodecylsulfate polyacrylamide gel
electrophoresis (159,160,161). It contains 24% carbohydrate by weight. This enzyme is activated by apoA-I but is inhibited by apoA-II (159,162).

The HDL₃ density subclass, rather than HDL₂, is the preferred substrate for LCAT (153). In fact, the concentration of HDL₂ is negatively correlated with LCAT activity in vitro (154). In vitro incubation of HDL₃ in the presence of LCAT results in a shift in HDL flotation rate and density consistent with a conversion of HDL₃ particles into HDL₂-like particles (151). Incubation of human plasma in vitro resulted in a decrease in the concentration of (HDL3)gge subpopulations and an increase in (HDL2b)gge (152).

Radioimmunoassay of human plasma LCAT in normolipidemic subjects shows that females have a higher LCAT level than males. There is an inverse relationship between HDL concentration and LCAT activity for females but not males (158).

Recently, the LCAT enzyme has also been shown to acylate lysolecithin, forming lecithin in the presence of LDL (155-157). The substrate source of the fatty acid for the lysolecithin acyltransferase reaction is not well characterized.

There are other metabolic enzymes besides LCAT that affect the HDL composition and physical-chemical properties. Hepatic triacylglycerol lipase has been reported to bind HDL (165). In vivo suppression of hepatic
lipase activity by administration of antibodies leads to an 
increase in the phospholipid composition of HDL₂ 
(166-168); the HDL₃ cholesteryl ester concentration 
decreases (166). Incubation of the phospholipid-enriched 
HDL₂ particles with the isolated hepatic lipase results 
in hydrolysis of phosphatidylcholine and 
phosphatidylethanolamine of the lipoprotein and formation 
of a cholesteryl ester-rich, HDL₂-like product (167). 

A variety of in vitro studies have suggested 
additional possibilities for the in vivo function of HDL. 
Elastase activity has been associated with apoA-I (163). 
The HDL reportedly inhibit the pyrogenic activity of 
bacterial lipopolysaccharides (164).

As previously mentioned, HDL also participate in the 
transfer and exchange of lipid components with other 
lipoproteins. In addition, a number of in vitro studies 
have shown a decrease in cellular cholesterol during 
incubations in the presence of HDL (169,170), presumably 
due to uptake of cholesterol by these lipoproteins (171). 
The reduction in cellular cholesterol, during incubations 
with HDL, results in an increased level of LDL receptors in 
fibroblasts (171). The HDL can also take up cholesterol 
dispersed on Celite (172), as well as from the crystalline 
form of this sterol (173,174). These in vitro observations 
support an anti-atherogenic role for HDL, wherein they 
participate in cholesterol removal from cells and in 
cholesterol transport to the liver for secretion as bile
acids (149,150). Indeed, recent data suggest that HDL are the primary cholesterol sources for bile acid production (175).

**H. METABOLIC INTERRELATIONSHIPS AMONG LIPOPROTEIN SPECIES**

It is clear from the brief discussion above that a variety of metabolic processes involve LDL or HDL. Metabolic interrelationships, particularly the transfer and exchange of lipid components, also exist between these lipoprotein classes. Metabolic interrelationships between HDL and either CM or VLDL have also been examined. Early studies have shown an inverse relationship between the plasma concentrations of VLDL and HDL and, therefore, have suggested a metabolic link between these lipoproteins (176). The possible physiological bases for these findings have only recently been explored.

**In vivo** studies on the acute clearance of VLDL by heparin-induced lipolysis have described a concomitant increase in mass within the HDL density range (177-179). Both **in vivo** and **in vitro** studies have reported the transfer of C apolipoproteins (46,180-182,188,190,192) and phospholipids (183) to the HDL density range during heparin-induced metabolism of VLDL. Also, **in vivo** catabolism of CM is associated with transfer of apolipoproteins (184-187) and phospholipid (184,186,187) to the density range of HDL.
These studies suggest that components arising from degradation of triacylglycerol-rich lipoproteins, CM and VLDL, interact with pre-existing HDL. The processes that mediate such an interaction have not been extensively characterized. A number of catabolic products have been described that may participate in the transfer of components from either CM or VLDL to HDL. Phospholipid vesicles have been reported during catabolism of triacylglycerol-rich lipoproteins (50,51,187). In addition, the in vitro triacylglycerol lipolysis of VLDL, performed in the absence of HDL, results in the appearance of apolipoprotein (48,49,188,189) and phospholipid (48,49,189) components within the density range of HDL. These phospholipid and apolipoprotein components form discoidal-shaped complexes (48,50,189). Finally, electron microscopy of CM undergoing lipolysis has demonstrated alterations in the lipoprotein shape and bilayered lamellae at the particle surface (195-197). Thus, surface components are released from triacylglycerol-rich lipoproteins in the form of phospholipid vesicles, apolipoprotein-phospholipid discoidal complexes, or both structures.

These findings are consistent with the view that catabolism of triacylglycerols in the lipoprotein core and removal of the products (fatty acids, mono-, and diglycerides) results in a decreased core volume and, consequently, in release of surface components,
phospholipids and apolipoproteins, that are in excess of those necessary for covering the smaller core.

Several *in vitro* (193) and *in vivo* studies (191, 224) have specifically examined the physical-chemical properties of HDL to determine whether the apolipoprotein and phospholipid components released during lipolysis of CM or VLDL interact with HDL. While these investigations have indicated changes in HDL properties, they did not characterize which of the possible surface catabolic products (vesicles, discoidal complexes, or both) might be responsible for the observed physical-chemical changes.

I. INCUBATIONS OF MODEL CATABOLIC PRODUCTS WITH HDL AND LDL

The nature and extent of interaction of HDL with catabolic products, formed during lipolysis of triacylglycerol-rich lipoproteins, have been investigated by a number of *in vitro* studies that utilized model products. The interaction of HDL$_{2b}$ with discoidal complexes of apoA-I and dimyristoylphosphatidylcholine (DMPC) has been investigated in a recent study (194). This investigation has demonstrated uptake of phospholipid from these complexes by HDL. The DMPC molecule, however, is a non-physiological phospholipid; therefore, the interaction study may not be representative of interactions of HDL with discoidal complexes composed of phospholipids that have long-chain, unsaturated fatty acids.
Early in vivo and in vitro studies on the interaction of radiolabeled liposomes of egg phosphatidylcholine with rat plasma have shown uptake of the radioactivity by a plasma component that was similar in chromatographic elution properties to HDL (198). For DMPC, a number of investigators have demonstrated that the interaction of human HDL with liposomes resulted in a net uptake of phospholipid by HDL (201) and in formation of discoidal complexes of apoA-I and DMPC (199-201); the change in HDL composition was associated with an increase in diameter (199-201) and a decrease in density for HDL (200).

Studies, utilizing liposomes composed of more physiologically relevant phospholipids (those with fatty acids of longer chain length and a greater degree of unsaturation than DMPC), have described a disruption of the bilayer that was sufficient to allow release of small entrapped molecules during the course of in vivo (204,208,211) or in vitro (203,204,209,210,212,216,218-220) interaction with blood components. Incorporation of cholesterol into the liposomes stabilizes them against disruption (203,204-206,209-211,218,219). Liposomes composed of DMPC are also disrupted during incubation with plasma (202).

The disruption of liposomes during these incubation studies might have been due, at least in part, to phospholipid uptake by HDL, as indicated by data for HDL from bovine (207), rat (211), and human (214,216,217,53-55).
sources. In addition to phospholipid uptake by HDL, recent findings demonstrate the formation of discoidal phospholipid-apolipoprotein complexes during incubation of vesicles with HDL (54,55). Since the phospholipid component of these discoidal complexes is contributed by vesicles (55), the formation of discoidal complexes may also result in liposome dissolution. Discoidal complexes can co-elute with HDL during column chromatography (55) because of the similarity in their dimensions and the relatively poor size resolution of this technique. Thus, previous studies that have relied on this fractionation procedure to assess uptake of phospholipid by HDL must be considered with this limitation in mind (205,213,214,216).

In contrast to the relatively large amount of work investigating the interaction of HDL with discoidal complexes and vesicles, the interaction of model catabolic products with LDL has not been studied extensively (222,223). One investigation has characterized the interaction of LDL with dipalmitoylphosphatidylcholine (DPPC) vesicles in the presence of a phospholipid exchange protein (222). Under these conditions, net uptake of phospholipid by LDL occurs and results in a 16 to 46% increase in phospholipid content after a 20 minute incubation. Recently, I have examined the interaction of single bilayer vesicles of egg PC with LDL in the absence of a phospholipid exchange protein (223). These studies have shown that LDL aggregates with vesicles.
**J. STATEMENT OF PURPOSE**

The focus of this thesis is on the interaction of plasma lipoproteins with phosphatidylcholine vesicles. The aims of these studies have been the following:

1) Develop a simplified model system for investigating the types of interactions which may be occurring in vivo between plasma lipoproteins, particularly LDL and HDL, and catabolically produced liposomes.

2) Investigate the effects of vesicle interaction on the physical-chemical properties of plasma lipoproteins.

3) Gain additional insight into the molecular structure of LDL and HDL by detailed characterization of the products formed during interaction with vesicles.

Consequently, these findings have stressed the physical-chemical characterization of lipoproteins and interaction products. Additionally, based on my findings, a framework can be developed within which the in vivo origins of the observed physical-chemical heterogeneity for these lipoprotein classes can be addressed. A thorough knowledge of the physical-chemical properties of lipoproteins and their metabolic modulation will be of importance for understanding the role of lipoproteins in atherogenesis, as well as, for assessing individual risk to cardiovascular disease.
II. MATERIALS AND METHODS

A. PLASMA ISOLATION

Blood was collected, using either heparin (7 units per ml of blood) or dipotassium ethylenediaminetetraacetic acid (K₂EDTA, 2.5 mM) as an anticoagulant, from normal subjects after an overnight fast. Plasma was separated by previously described centrifugal methods (23). Two mM p-chloromercuriphenylsulfonic acid (Sigma) was added as a lecithin:cholesterol acyltransferase (E.C. 2.3.1.43) inhibitor (149,150). In addition, 2 mM phenylmethylsulfonyl fluoride (Sigma) was added as a protease inhibitor to plasma used for LDL isolation.

B. ISOLATION OF PLASMA LIPOPROTEINS

For isolation of subclasses of high density lipoproteins (HDL), aliquots of plasma were adjusted by addition of solid NaBr to a density of either 1.071 g/ml for isolation of HDL₂b, 1.106 g/ml for isolation of HDL₂a, or 1.125 g/ml for isolation of HDL₃. (Solution densities were determined using a precision Abbe refractometer.) After ultracentrifugation in a Beckman 50.3 rotor (114,568 x g) for 36 h at 4 °C, the top 2 ml were
removed from the samples that were adjusted to 1.071 and 1.106 g/ml. The top 3 ml were removed from the sample adjusted to 1.125 g/ml. The infranatants of the ultracentrifuged 1.071, 1.106, and 1.125 g/ml plasma samples were separately dialyzed against solutions of densities 1.106, 1.125, and 1.210 g/ml, respectively. All dialysis solutions contained 0.195 M NaCl, 0.27 mM Na₂EDTA, 0.12 mM ethylmercurithiosalicylic acid, and sufficient NaBr to give the desired solution density. A second ultracentrifugation was performed by overlaying 3 ml of the plasma infranatants with 3 ml of the corresponding salt solution used for dialysis. No convection was apparent under these conditions because the density of plasma was actually greater than the salt density in which it was dialyzed due to the contribution of plasma proteins to the total solution density. The subclasses HDL₂b, HDL₂a, and HDL₃ were then recovered from the top 2 ml following ultracentrifugation (114,568 x g, 36h, 4 °C) of the plasma at 1.106, 1.125, and 1.210 g/ml, respectively, and were used without additional ultracentrifugation.

Total HDL was isolated by ultracentrifugation from an aliquot of plasma in a sequential manner similar to that described for HDL subclasses but at densities of 1.071 and 1.210 g/ml.

All HDL samples were dialyzed against the incubation buffer (pH 7.2, 0.01 M Tris (Sigma), 0.195 M NaCl (Mallinckrodt), 0.27 mM Na₂EDTA (Sigma), 0.12 mM...
ethylmercurithiosalicylic acid (Aldrich Chemical Co.) and stored in screw-cap glass vials under N₂ at 4 °C.

Gradient gel electrophoresis of the isolated HDL subclasses (Figs. 6A-8A) and total HDL showed bands confined to the previously defined intervals within the HDL migration range (34). Gradient gel electrophoresis was performed on all isolated HDL samples and showed no evidence of contamination from albumin or other plasma proteins.

Low density lipoproteins (LDL) were also isolated from plasma by sequential ultracentrifugation. The first ultracentrifugation (24 h, 114,568 x g, 17 °C) was performed after adjusting plasma to a background solution density of 1.019 g/ml with solid NaBr. The top 2 ml were removed, and the bottom 4 ml were adjusted to a density of 1.063 g/ml by addition of 2 ml of 1.1483 g/ml (0.195 M NaCl, 1.937 M NaBr) salt solution. After a second ultracentrifugation (24 h, 114,568 x g, 17 °C), the top 2 ml, containing LDL (d 1.019-1.063 g/ml), were removed and stored under N₂ at 4 °C in a screw-cap glass vial.

Column chromatography of LDL was subsequently performed to remove any contaminating lipoprotein(a) (Lp(a)) species, which exhibit densities in the 1.050 to 1.120 g/ml range (226). Gel filtration column chromatography was previously shown to be an effective method of separating LDL from Lp(a) based on their differences in particle diameter (228). Five ml of the ultracentrifugal fraction, d 1.019-1.063 g/ml, were applied
to a column (2.5 x 100 cm, Bio-Gel A-5M, Bio-Rad Laboratories) and eluted with the incubation buffer used for studies of LDL (0.05 M Tris, 0.195 M NaCl, 0.27 mM Na$_2$EDTA, 0.12 mM ethylmercurithiosalicylic acid, 1.6 mM reduced glutathione (Calbiochem)). When monitored spectrophotometrically at 280 nm, LDL eluted as a single symmetrical peak. Purified LDL were obtained by pooling the 2 fractions (4 ml/fraction) just prior to the peak maximum absorbance and all subsequent fractions exhibiting an absorbance above the baseline value. This pool was then concentrated under N$_2$ pressure by ultrafiltration (Amicon Corp., XM100A) to obtain a phospholipid concentration for LDL (LDL-PL) of approximately 2 mg/ml. Gradient gel electrophoresis of the isolated LDL did not exhibit any protein or lipid-staining bands characteristic of Lp(a). Only bands in the LDL migration range were observed (see Fig. 25A, lane 1).

C. EXTRACTION AND ISOLATION OF PHOSPHATIDYLCHOLINE

Human phosphatidylcholine (PC) was extracted from plasma and packed erythrocytes by the methods of Bligh and Dyer (228) and of Rose and Oaklander (229), respectively. The total lipid extract was dried using rotary evaporation under partial vacuum and stored in chloroform under N$_2$ at -20 °C.

For chromatographic separation of PC, 50 gm of
Figure 1. Isolation of phosphatidylcholine by column chromatography. Approximately 1 g of phospholipid in a total lipid extract from human plasma was applied to a silicic acid (50 g) column (2.95 x 18 cm). The column was initially eluted with 400 ml of a 4:1 (v:v) chloroform:methanol solvent and, subsequently, eluted with 800 ml of a 1:1 chloroform:methanol solvent. Twenty-five ml fractions were collected during elution with the latter solvent. Phospholipid concentration was determined chemically (241).
activated (115 °C, 2 h) silicic acid were packed in a column (Unisil, 200-325; Biosil HR, -325) and pre-treated by elution with 320 ml of methanol followed by 320 ml of chloroform. The lipid extract, containing at most 1 g of total phospholipid, was applied to the column and fractionated by a modification of the method of Williams et al. (230). Specifically, the column was eluted with 400 ml of a 4:1 (v:v) chloroform:methanol solvent and then with 800 ml of a 1:1 chloroform:methanol solvent mixture; 25 ml fractions were collected. A typical elution pattern (Fig. 1) showed a major peak and a broad trailing shoulder.

Analytical thin layer chromatography (Redi-Coat G, 0.250 mm plates, Supelco, Inc.) was used to identify fractions containing pure PC. The 25 ml solvent volume of each fraction was reduced to 2 ml under a stream of N₂. The lipid mass applied to each lane was at least 200 μg for fractions containing PC. Two solvent mixtures (A and B) were initially investigated for establishing optimal lipid separation on the thin layer chromatography (TLC) plates used. Lipids were visualized by spraying the plates with 50% aqueous sulfuric acid followed by charring on a hot plate. The average Rf values for sphingomyelin (SM) and for polar lipid standards (Supelco, Inc.) of unesterified cholesterol (UC), phosphatidylethanolamine (PE), phosphatidylcholine (PC), and lysophosphatidylcholine...
Table II. THIN LAYER CHROMATOGRAPHY OF LIPIDS

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Solvent A†</th>
<th>Solvent B++††</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPC</td>
<td>0.049 ± 0.003</td>
<td>0.101 ± 0.029</td>
</tr>
<tr>
<td>SPH</td>
<td>0.105 ± 0.014</td>
<td>0.153 ± 0.025</td>
</tr>
<tr>
<td>PC</td>
<td>0.177 ± 0.017</td>
<td>0.287 ± 0.028</td>
</tr>
<tr>
<td>PE</td>
<td>0.454 ± 0.039</td>
<td>0.706 ± 0.037</td>
</tr>
<tr>
<td>UC</td>
<td>0.833 ± 0.043</td>
<td>1.000 ± 0.000</td>
</tr>
</tbody>
</table>

*Mean standard deviation of Rf values (ratio of migration distances of solvent front to lipid band) are reported.

†Abbreviations are the following: lysophosphatidylcholine, LPC; sphingomyelin, SPH; phosphatidylcholine, PC; phosphatidylethanolamine, PE; unesterified cholesterol, UC.

††Chloroform:Methanol:Water, 80:35:5, v:v:v

(LPC) are presented in Table II. Solvent mixture A was chloroform:methanol:water (80:35:5, v:v:v), and solvent mixture B was chloroform:methanol:acetic acid:water (150:90:24:12, v:v:v:v). Solvent mixture B provided a better separation of PC and SM and was used in subsequent analyses of lipid samples.

Thin layer chromatography of the fractions from the silicic acid column showed that pure PC (fractions 1-7) formed the main elution peak and eluted prior to fractions comprising the trailing shoulder, which contained a mixture of PC and SM (fractions 8-16). Fractions containing pure PC (fractions 1-5) were pooled; fractions collected just prior to the appearance of SM were excluded from the pool. The elution profile and polar lipid elution sequence agree well with previously reported data (230).

Egg yolk PC was obtained either from commercial sources (U.S. Biochemical Corp. and Gibco) or isolated from fresh egg yolks. If analytical TLC of commercial PC samples revealed contaminating lipids, then purification was performed by silicic acid chromatography, as detailed above for human PC isolation. Preparation of purified egg PC from isolated yolks was performed by lipid extraction using the method of Bligh and Dyer (228) and silicic acid chromatography as described for human PC.
D. FORMATION AND ISOLATION OF SMALL UNILAMELLAR VESICLES

Small unilamellar vesicles were produced by a modification of the method of Huang (231). Purified PC (100 mg) was dried in a sonication vial under a stream of N₂. The PC was then left overnight in a dessicator under vacuum to remove any residual solvent. Sonication (Sonifier Cell Disruptor, Heat Systems-Ultrasonics, Inc.) of PC with 5 ml of incubation buffer was performed, under N₂ and on ice, using a 1.2 cm diameter probe tip. Five minutes of sonication were followed by 5 minutes of temperature re-equilibration to 4 °C. The cumulative sonication time was based on attainment of a minimum for absorbance readings at 300 nm. For preliminary studies at low concentrations of lipid, readings of 2.525, 1.078, 0.598, 0.515, and 0.511 were obtained for sonication of 1.8 mg/ml PC at 30 seconds, 5 min, 10 min, 15 min, and 20 min, respectively. A cumulative sonication time of 30 min produced a minimum absorbance value which was associated with maximal yield of small unilamellar vesicles (Fig. 28B). The temperature of the sonicated solution was found to be less than 25 °C immediately after the last 5 minute sonication period. The lipid dispersion was re-equilibrated to the ice bath temperature, and ultracentrifuged (Beckman 50.3 rotor, 1 h, 84,988 x g, 4 °C) to sediment contaminating titanium particles released from the probe tip and some large multilamellar lipsomes.
Figure 2. Fractionation of sonicated phosphatidylcholine by column chromatography. The sonicated phosphatidylcholine mixture (approximately 70 mg of PC) was applied to a column (2.5 x 50 cm) containing Bio-Gel A-5M and eluted with either the HDL incubation buffer (0.01 M Tris, 0.195 M NaCl, 0.27 mM Na₂EDTA, and 0.12 mM ethylmercurithiosalicylic acid) or the LDL incubation buffer (0.05 M Tris, 0.195 M NaCl, 0.27 mM Na₂EDTA, and 0.12 mM ethylmercurithiosalicylic acid, 1.6 mM reduced glutathione). The absorbance (280 nm) was monitored continuously during fractionation (4 ml/fraction). Multilamellar liposomes eluted at the void volume, Vo (fractions 18-22), and small unilamellar vesicles eluted as a single trailing peak (fractions 26-42). Vesicles used for incubations were a pool of fractions 33-42. The elution of carboxyfluorescein (fractions 60-68) not trapped within vesicles is also shown.
The supernatant was removed and stored under N₂ in screw-cap glass vials.

Small unilamellar vesicles (SUV) were isolated by gel filtration chromatography (Bio-Gel A-5M, 2.5 cm x 50 cm). A representative elution pattern (Fig 2) was similar in shape to that previously described (231). Multilamellar liposomes eluted at the void volume (Vo). Small unilamellar vesicles eluted in a single peak over fractions 26 to 42. The stock solution of small unilamellar vesicles, used for incubation studies, comprised a pool of two fractions just prior to the absorbance maximum and all subsequent fractions exhibiting an absorbance above the baseline value (i.e., fractions 34 to 42 of Fig. 2). This pool was concentrated by ultrafiltration under N₂ (XM100A membrane, Amicon, Inc.). Electron microscopy using negative staining (Fig. 28B) showed a homogeneous population of collapsed vesicles forming rouleaux. The dimensions of the long and short axes for individual collapsed vesicles were 27.3 ± 3.3 and 11.9 ± 2.0 nm (mean ± SD), respectively. Previous studies indicate that vesicle collapse and rouleaux formation are artifacts of negative staining and dehydration and that electron micrographs do not represent the solution morphology for vesicles (232). Vesicles were stored under N₂ at 4 °C and used for incubation studies within a few days of their isolation. Chromatography of the isolated SUV after storage for three weeks (maximum time examined) only showed
a single peak which was identical in elution volume to that obtained during the initial isolation. Thus, large changes in the vesicle diameter resulting from vesicle-vesicle fusion or aggregation were not evident during this storage period.

E. INCUBATION CONDITIONS FOR LIPOPROTEIN-VESICLE STUDIES

Each density subclass of HDL, HDL$_3$, HDL$_{2a}$ and HDL$_{2b}$, was separately incubated with varying amounts of added vesicle phosphatidylcholine (SUV-PC) for 6 and 24 h at 37 °C under N$_2$ in screw-cap glass vials. The phospholipid concentration of HDL (HDL-PL) in the incubation mixtures was in the range of 0.5-1.0 mg/ml and approximated the plasma concentration of phospholipid transported by this lipoprotein class. The weight ratios of vesicle PC to HDL phospholipid (SUV-PC:HDL-PL) in the incubation mixtures were in the range of 0:1 to 4:1 for each subclass. All reactants were in a buffer that comprised 0.01 M Tris, 0.195 M NaCl, 0.27 mM Na$_2$EDTA, 0.12 mM ethylmercurithiosalicylic acid, pH 7.2.

Studies involving LDL were usually performed at a constant phospholipid concentration of this lipoprotein (LDL-PL) in the range of 0.5-0.75 mg/ml -- similar to normal plasma levels of LDL-PL. The LDL were separately incubated (37 °C, under N$_2$) with SUV-PC at weight ratios (SUV-PC:LDL-PL) in the range of 0:1 to 4:1.
Figure 3. Density gradients for fractionation of HDL incubation mixtures. The density gradients are shown before (solid line) and after (dashed line) ultracentrifugation (36 h, 186,029 x g, 15 °C). One ml fractions were collected after ultracentrifugation. Densities of fractions that did not contain lipoproteins were determined by a precision Abbe refractometer.
Studies examining the effect of temperature on LDL-vesicle reaction kinetics were conducted at a constant weight ratio (SUV-PC:LDL-PL), either 1:1 or 2:1, and at various incubation temperatures. The incubation times depended on the type of experiment being conducted. All reactants for LDL incubation studies were in buffer (LDL incubation buffer) of 0.05 M Tris, 0.195 M NaCl, 0.27 mM Na₂EDTA, 0.12 mM ethylmercurithiosalicylic acid, and 1.6 mM reduced glutathione at pH 7.2, unless otherwise noted. All buffers were prepared using de-ionized distilled water (Millipore).

Incubation studies for other plasma lipoproteins, Lp(a), IDL, and VLDL, were performed at 37 °C using the LDL incubation buffer.

F. ISOLATION OF INTERACTION PRODUCTS FROM INCUBATION MIXTURES

The HDL incubation mixtures were fractionated by equilibrium density gradient ultracentrifugation; 1 ml of each incubation mixture was applied to a step density gradient formed with NaBr salt solutions containing 0.195 M NaCl (Figures 3 A-D). For HDL₃, the sequence of solutions forming the step gradient (from bottom to top) were 2 ml of 1.4740 g/ml, 1 ml of sample adjusted with solid NaBr to 1.1450 g/ml, 2 ml of 1.1163 g/ml, and 7 ml of 1.0400 g/ml. For HDL₂a, the sequence of the step gradient was 3 ml of
1.1163 g/ml, 1 ml of sample adjusted to 1.0800 with solid NaBr, 4 ml of 1.0706 g/ml, and 4 ml of 1.0490 g/ml. For HDL$_{2b}$, the step gradient was 2 ml of 1.1002 g/ml, 1 ml of sample adjusted to 1.0900 g/ml with solid NaBr, 6 ml of 1.0700 g/ml, and 3 ml of 1.0490 g/ml. An additional gradient (Fig. 3D) was used for studies involving HDL$_{2b}$ in order to subfractionate products rich in phospholipid. This gradient was formed by layering the following sequence of salt solutions: 4 ml of 1.0680 g/ml, 1 ml of sample adjusted to 1.058 g/ml with solid NaBr, 4 ml of 1.0480 g/ml, and 3 ml of 1.0380 g/ml. Ultracentrifugation was performed using a Beckman SW 41 rotor (36 h, 186,029 x g, 15°C). One ml fractions were sequentially pipetted down the tube and stored under N$_2$ at 4°C in screw-cap glass vials until analyzed for protein and phospholipid.

LDL incubation mixtures were fractionated by velocity ultracentrifugation. Initially, this was accomplished by layering 1 ml of an incubation mixture (d 1.006 g/ml) over 5 ml of 1.019 g/ml and 6 ml of 1.032 g/ml salt solutions. However, better separation of incubation products was subsequently obtained using one ml of incubation mixture layered over a linear preformed salt gradient ranging from 1.019 to 1.030 g/ml. The gradient was formed using a peristaltic pump and a homemade gradient maker. The two salt solutions used had densities of 1.0188 and 1.0297 g/ml (prepared with solid NaBr and a solution of 0.195 M NaCl). Ultracentrifugation was performed in a Beckman SW-41 rotor.
Figure 4. Absorption spectra of HDL, LDL, d>1.200 g/ml plasma fraction, and beta-carotene. The ordinate is in arbitrary absorbance units. A) HDL in incubation buffer (see legend of Fig. 2 for HDL buffer). B) LDL in incubation buffer (see legend of Fig. 2 for LDL buffer). C) beta-carotene in chloroform. D) d>1.200 g/ml plasma fraction in HDL incubation buffer.
G. SPECTROPHOTOMETRY OF LDL INCUBATION MIXTURES

Spectrophotometry (Varian Super Scan III) at 340 nm and a slit width of 5 nm was performed on incubation mixtures of LDL and vesicles to monitor the formation of light-scattering products. Prior to spectrophotometry, samples incubated at temperatures below 20 °C were briefly centrifuged (3 min, 2000 rpm) in a 4 °C refrigerated centrifuge (Model PR-2 Centrifuge, International Equipment Co.) to reduce light scattering arising from macroscopic flocculent particles or dust. Samples incubated above 20 °C were briefly centrifuged (3 min, 2000 rpm) at room temperature (approximately 23 °C, Model J-6B Centrifuge, Beckman). After centrifugation, samples were placed in their respective temperature-controlled water baths and kept at the incubation temperature until they were analyzed in quartz cells.

The absorption spectra for unincubated lipoproteins (Figs. 4 A,B) were used to select the 340 nm wavelength for monitoring the turbidity of incubation mixtures. The multi-peaked spectra for lipoproteins in the range of 400 to 550 nm was probably due to their content of beta-carotene (233). The absorption peaks were similar to those for beta-carotene (Fig. 4C). For comparison, the
absorption spectra for the plasma components at d>1.200 g/ml are shown in Figure 4D. The peak at 417 nm is probably due to bilirubin, which has an absorption peak at the same wavelength (234); bilirubin is carried by albumin (235). Spectrophotometry at 340 nm was chosen to minimize lipoprotein absorbance due to either apolipoproteins or carotenoids, as well as to maximize any light scattering from LDL-vesicle products.

H. ELECTROPHORETIC PROCEDURES

Slab gel electrophoresis was performed using a Tris-borate buffer (0.09 M Tris, 0.08 M borate, 0.003 M Na₂EDTA, pH 8.35) as previously described (34). Linear 4 to 30% polyacrylamide gradient gels (PAA 4/30, Pharmacia Fine Chemicals) were used for electrophoresis of HDL incubation mixtures, and 2 to 16% polyacrylamide gradient gels (PAA 2/16, Pharmacia Fine Chemicals) were used for LDL incubation mixtures. All slab gels were prepared by electrophoresis for at least 15 minutes at 75 volts (V) prior to adding incubation mixtures. Samples (10-15 μl) were mixed with a 5 μl aliquot of a 40% sucrose solution containing bromophenol blue (0.05%) and an internal standard of thyroglobulin (1.75 mg/ml). Ten to 15 μl of the mixture were applied to the sample well of the gel to give about 20 μgm of protein. Lanes of the gel that did not contain a lipoprotein sample were filled with an
equivalent mixture of incubation buffer and sucrose. The electrophoretic voltage was gradually increased in the following manner: 15 V for 15 min, 70 V for 20 min, and 125 V for 24 hours. Gels were stained using a perchloric acid and Coomassie G-250 (Isolab Inc.) mixture and destained by diffusion using 5% aqueous acetic acid (236).

Scans of gradient gels for samples of LDL and HDL were obtained by densitometry (RFT Scanning Densitometer, Transidyne General Corp.), and particle sizes of lipoproteins were obtained by computer analysis (PDP 8/e, Digital Equipment Corp.) as previously described (34). For 4-30% gradient gels, protein standards (High Molecular Weight Calibration Kit, Pharmacia Fine Chemicals) of thyroglobulin (17.0 nm diameter), apoferritin (12.2 nm), lactate dehydrogenase (8.2 nm), and bovine serum albumin (7.1 nm) were used to obtain a best-fit quadratic regression curve of radius versus Rf. In this case, Rf is defined as the ratio of the migration distance for the sample peak to the migration distance for the bovine serum albumin peak. The hydrated diameters were computed from the available hydrodynamic data. For 2-16% gradient gels, the same protein standards were applied. In this case, however, lactate dehydrogenase and bovine serum albumin migrated off the gel. Carboxylated latex beads (38.0 nm diameter, Dow Diagnostics) were also added to the standard mixture to expand the range of the calibration curve. Areas under densitometric peaks were obtained by computer
integration.

For HDL samples, apolipoprotein composition was determined, after delipidation with tetramethylurea, using polyacrylamide disc electrophoresis as previously described (237). Apolipoprotein identity was determined by electrophoresis of total HDL apolipoproteins and by using established data on the relative electrophoretic migration of the apolipoproteins (238). Gels were stained and destained as described above for the slab gels. Scanning densitometry and computer integration of peaks were performed to estimate the relative concentration of the apolipoproteins.

For LDL, sodium dodecylsulfate (SDS) electrophoresis of the apolipoprotein was performed by a modification of the method of Weber and Osborn (239) on 4% polyacrylamide gels. Apolipoprotein delipidation was accomplished by the method of Bligh and Dyer (228). Staining and destaining were accomplished as described above for the slab gels.

I. ELECTRON MICROSCOPY

Samples were dialyzed in a solution of 0.126 M ammonium acetate and 0.003 M ammonium carbonate at pH 7.4. An aliquot of sample was mixed with an equal volume of 2% sodium phosphotungstic acid (pH 7.4). A droplet of this mixture was applied to a carbon coated Formvar grid for 30
seconds, and excess fluid was removed subsequently with filter paper. The sample was examined using an electron microscope (JEM 100C, JEOL., Inc.) that operated at an accelerating voltage of 100 KeV. Micrographs were obtained at an instrument magnification in the range of 20,000 to 60,000. An additional 3 to 5 fold magnification occurs when the electron micrographs were printed.

J. CHEMICAL ANALYSES

Protein and phospholipid concentrations were determined by chemical methods described previously (240, 241). In order to eliminate turbidity due to vesicles, SDS was added to aliquots of turbid fractions to give a final concentration of 0.12%. All other samples were assayed without detergent. Bovine serum albumin was used as a protein standard, with addition of sodium dodecylsulfate when it was appropriate. Total cholesterol was determined enzymatically (Cholesterol Reagent Set, Boehringer-Mannheim). The concentration values reported for chemical components are the means of at least duplicate measurements.
K. OSMOLARITY DETERMINATION

To avoid osmotic perturbation of the vesicles, the osmolality of all buffers was routinely checked using an osmometer (Fiske) calibrated with aqueous NaCl standards (Fiske). For investigations using carboxyfluorescein (CF) trapped within vesicles, the incubation buffer was adjusted with solid NaCl to match the osmolality of the CF solution to within ± 3 mOsm.

L. CALCULATIONS

For ultracentrifugal fractionation data of HDL, there was some overlap of incubation products in the density gradient fractions at the two highest incubation ratios examined. A best estimate of the relative contribution of the two overlapping products was made using the following expressions:

1) \( PT = P_1 + P_2 \), where \( PT \) represents the protein mass in the overlap region; \( P_1 \) and \( P_2 \) are the protein masses contributed by products 1 and 2 in the overlap region, respectively.

2) \( LT = L_1 + L_2 \), where \( LT \) represents the phospholipid mass in the overlap region; \( L_1 \) and \( L_2 \) are the phospholipid masses contributed by products 1 and 2 in the overlap region, respectively.

3) \( R_1 = \) phospholipid/protein weight ratio for product 1.
4) \( R_2 = \frac{\text{phospholipid/protein weight ratio for product 2}}{} \).

5) \( P_1 = \frac{P_T - (LT/R_2)}{1 - (R_1/R_2)} \)

The corrected data obtained did not alter the distribution analysis of protein or phospholipid among the incubation products by more than 5%.

M. DETERMINATION OF VESICLE INTEGRITY

Leakage through the vesicle bilayer during incubations of LDL with vesicles was monitored by entrapping either carboxyfluorescein (MW 377.3, Eastman Kodak) or \(^3\)H-Inulin (MW approximately 5000, Amersham Corp.). These probes have been previously used for monitoring vesicle integrity during interaction with cells and plasma components (242,243).

Carboxyfluorescein (CF) was purified according to the method of Weinstein et al. (244) to remove apolar impurities. Fifty ml of carboxyfluorescein (200 mM) was allowed to completely enter a column containing 50 g of Sephadex LH-20 (Pharmacia Fine Chemicals). The sample was eluted with de-ionized distilled water. The 1st, 2nd, and 3rd fractions (30 ml/fraction) of CF were collected and analyzed by TLC (Fig. 5A). The apolar impurities (those bands that tended to migrate with the solvent front) were removed by the column chromatography technique. The absorption spectra for purified CF is shown in Figure 5B.
Figure 5. Thin layer chromatography and absorption spectrum of purified carboxyfluorescein (CF). Purification of CF was performed by column chromatography (Sephadex LH-20) as previously described (244); 30 ml fractions were collected during elution of the column with de-ionized distilled water.

A. Analytical thin layer chromatography (Sulpelco Redi-Coat G; developing solvent, chloroform : methanol : acetic acid : water, 150 : 90 : 24 : 12, v : v : v : v) of fractions (30 ml) collected during purification of CF. Lane 1, initial CF (10ul applied); lane 2, initial CF (20ul); lane 3, fraction 1 (10ul); lane 4, fraction 1 (20ul); lane 5, fraction 2 (10ul); lane 6, fraction 2 (20ul); lane 7, fraction 3 (10ul); lane 8, fraction 3 (20ul). Lanes 1,2 (10 ul, 20 ul applied, respectively); fraction 1, lanes 3,4 (10 ul, 20 ul applied); fraction 2, lanes 5,6 (10 ul, 20 ul applied); fraction 3, lanes 7,8 (10 ul, 20 ul applied).

B. Absorption spectrum of purified CF (fraction 1). Absorbance is in arbitrary units.
B

CARBOXYFLUORESCEIN

492
The purified sample was concentrated to about one third of its volume to give a concentration of 114 mM based on the extinction coefficient of $58 \times 10^3$ optical density per mole at 492 nm reported for the purified molecule (244). At this concentration it is predominantly self-quenched (242).

Five ml of the CF solution were added to 100 mg of PC and vesicles were formed and isolated by the sonication and chromatography procedures previously described. The elution buffer for column chromatography was adjusted with solid NaCl to match the osmolarity of the CF solution (641 mOsm). Separation of untrapped CF from vesicles containing trapped CF was accomplished during the chromatographic isolation of small unilamellar vesicles (Fig. 2).

Measurement of CF leakage from vesicles was performed by diluting 10 μl aliquots in 10 ml of osmotically matched buffer and reading the fluorescence (MK 2 Spectrofluorometer, Farrand Optical Co.,Inc.) at an emission wavelength setting of 510 nm and an excitation wavelength setting of 490 nm. SDS was added to give a final concentration of 0.12% in the vesicle mixtures in order to obtain a fluorescence reading at 100% release of CF. Release of CF from vesicles is expressed as a percentage of the maximum detergent-induced release of CF. 

$^3$H-Inulin was also used to monitor perturbations of the vesicle bilayer during interaction with LDL. It was dissolved in the LDL incubation buffer to give a final
radioactivity per volume of 4.19 μC/ml buffer for the incubation weight ratio of 1:1 (SUV-PC:LDL-PL) and 17.26 μC/ml for the weight ratio of 2:1. Five ml of this solution were added to 100 mg of PC prior to sonic production of vesicles. Free inulin was separated from entrapped molecules during the chromatographic isolation of SUV.

Measurements of the release of radiolabeled inulin from vesicles during incubation with LDL were made by taking a 0.5 ml aliquot from the incubation mixture and performing ultrafiltration under N₂ pressure (XM50 membrane, Amicon Corp.) until at least 0.2 ml of effluent was collected. A 0.2 ml aliquot of effluent was then transferred to a scintillation vial and mixed with 25 ml of scintillation fluid (Ultrafluor, National Diagnostics). Liquid scintillation counting was performed on a Mark III, 6880 Liquid Scintillation System (Searle Analytic, Inc.), and the results were corrected for quenching using a calibration curve of variably quenched ³H standards (Amersham/Searle). The counting efficiency for all ³H-labeled samples was about 45%. 
III. RESULTS

A. CHARACTERIZATION OF ULTRACENTRIFUGAL HDL SUBCLASSES

In the present study, the ultracentrifugally-isolated density subclasses of HDL, HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b}, exhibited phospholipid to protein weight ratios similar to those previously described \(24\). The weight ratios were 0.425:1, 0.560:1, and 0.833:1 for HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b}, respectively.

The particle diameters, obtained by using gradient gel electrophoresis (GGE), of HDL size subpopulations within these subclasses were consistent with data from a recent GGE study of the frequency distribution of HDL particle sizes in a large number of human plasma samples \(34\). In the present study, single peaks were observed in GGE patterns of the ultracentrifugal HDL\textsubscript{2a} and HDL\textsubscript{2b} density subclasses (Figs. 7A, 8A); the corresponding diameters were 9.3 and 10.4 nm, respectively. These values were within the particle diameter ranges of 8.8 to 9.8 nm and 9.8 to 12.6 nm used to define the \((\text{HDL}_{2a})_{\text{gge}}\) and \((\text{HDL}_{2b})_{\text{gge}}\) size subpopulations, respectively \(34\). Thus, in subsequent discussions, these GGE components will be referred to as \((\text{HDL}_{2a})_{\text{gge}}\) and \((\text{HDL}_{2b})_{\text{gge}}\) size populations. The GGE pattern of HDL\textsubscript{3} (Fig. 6A) showed two peaks with corresponding particle diameters of 7.9 and...
Figure 6. Densitometric scans of stained gradient gels after electrophoresis of HDL3 incubation mixtures. Electrophoresis (10 °C) was performed for 24 h at 125 V on 4-30% polyacrylamide gradient gels (PAA 4/30, Pharmacia Fine Chemicals). The control and the highest weight ratio incubation mixtures are shown. Thyroglobulin was used as an internal protein calibration standard.

A. HDL3 (HDL3-PL, 0.705 mg/ml) incubated (37 °C) in the absence of vesicles for 6 h.

B. HDL3 (HDL3-PL, 0.705 mg/ml) incubated (37 °C) at a molar ratio of 295:1 (PC:HDL) for 6 h.
Figure 7. Densitometric scans of stained gradient gels after electrophoresis of HDL\textsubscript{2a} incubation mixtures. Electrophoretic conditions are the same as those described in the legend to Figure 6. The control and highest weight ratio incubation mixtures are shown.

A. HDL\textsubscript{2a} (HDL\textsubscript{2a}-PL, 0.385 mg/ml) incubated (37 °C) in the absence of vesicles for 6 h.

B. HDL\textsubscript{2a} (HDL\textsubscript{2a}-PL, 0.385 mg/ml) incubated (37 °C) at a molar ratio of 710:1 (PC:HDL) for 6 h.
Figure 8. Densitometric scans of stained gradient gels after electrophoresis of HDL<sub>2b</sub> incubation mixtures. Electrophoretic conditions are identical to those described in the legend to Figure 6. The control and highest weight ratio incubation mixtures are shown.

A. HDL<sub>2b</sub> (HDL<sub>2b</sub>-PL, 0.859 mg/ml) incubated (37 °C) in the absence of vesicles for 6 h.

B. HDL<sub>2b</sub> (HDL<sub>2b</sub>-PL, 0.859 mg/ml) incubated (37 °C) at a molar ratio of 1045:1 (PC:HDL) for 6 h.
8.6 nm. Multiple peaks were also observed for the ultracentrifugual \textit{HDL}_3 density subclass in the studies of Blanche et al (34). These investigators identified the following \textit{HDL}_3 size subpopulations, as determined by GGE: \(\text{HDL}_{3a}\)gge (8.2-8.8 nm); \(\text{HDL}_{3b}\)gge (7.7-8.2 nm) and \(\text{HDL}_{3c}\)gge (7.2-7.7 nm). Thus, in the present work the peak (7.9 nm particle diameter) in the GGE pattern of the \textit{HDL}_3 density subclass (Figure 6A) fell within the \(\text{HDL}_{3b}\)gge range, and the second peak (8.5 nm particle diameter) fell within the \(\text{HDL}_{3a}\)gge range. These GGE components will be separately referred to as \(\text{HDL}_{3b}\)gge and \(\text{HDL}_{3a}\)gge size subpopulations in subsequent discussions. Together they will be referred to as \(\text{HDL}_3\)gge size subpopulations.

\section*{B. ELECTROPHORETIC CHARACTERIZATION OF HDL-VESICLE INTERACTION MIXTURES}

Each density subclass of HDL was separately incubated (37 \textdegree C), at a constant concentration, with various amounts of vesicles, up to a maximum weight ratio of about 4:1 (SUV-PC:HDL-PL). The specific phospholipid concentrations of \textit{HDL}_3, \textit{HDL}_{2a}, and \textit{HDL}_{2b} density subclasses in the incubation mixtures were 0.705, 0.385, and 0.859 mg/ml, respectively. By calculation from compositional data for these HDL subclasses (24), the maximum molar ratios of phosphatidylcholine:HDLP}
(PC:HDL) in incubation mixtures containing HDL₃, HDL₂ᵃ, and HDL₂ᵇ were 295:1, 710:1, and 1045:1, respectively. Gradient gel electrophoresis was performed on each mixture, after incubation for 6 and 24 h, to determine the change in particle diameter of the HDL size subpopulations in each density subclass.

Representative, GGE patterns for each of the HDL density subclasses incubated for 6 h, at the highest weight ratio, are shown in Figs. 6B,7B,8B. The GGE patterns for HDL₂ᵃ and HDL₂ᵇ density subclasses indicate an increase in particle diameter when compared with the corresponding non-incubated HDL density subclass (Figs. 7,8). After incubation of the HDL₃ mixture, two subpopulations were still observed in the GGE pattern. However, there was a shift of the entire GGE pattern consistent with an increase in particle size of both HDL₃ subpopulations. There was no evidence of a subfraction of lipoprotein particles that did not increase in size. Thus, all lipoprotein particles constituting the HDL size subpopulations increased in diameter during incubation with vesicles.

In addition to the above changes in GGE patterns of the different HDL size subpopulations, several peaks appeared outside of the normal electrophoretic migration range of HDL species. The apparent particle sizes for these incubation products fell within the 24.0 to 31.0 nm diameter interval. The particle sizes observed for these incubation products were similar for all HDL density
Table III. PARTICLE SIZES OF NON-HDL COMPONENTS FROM GRADIENT GEL ELECTROPHORESIS*

<table>
<thead>
<tr>
<th>Incubation Ratio**</th>
<th>HDL$_3$ Apparent Diameter (nm)</th>
<th>Incubation Ratio**</th>
<th>HDL$_{2a}$ Apparent Diameter (nm)</th>
<th>Incubation Ratio**</th>
<th>HDL$_{2b}$ Apparent Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40:1</td>
<td>30.2, 26.6, 24.8</td>
<td>90:1</td>
<td>ND***</td>
<td>10:1</td>
<td>28.2, 25.5</td>
</tr>
<tr>
<td>85:1</td>
<td>30.7, 26.6</td>
<td>175:1</td>
<td>ND</td>
<td>290:1</td>
<td>28.0, 25.0</td>
</tr>
<tr>
<td>150:1</td>
<td>33.8, 29.9, 26.4</td>
<td>345:1</td>
<td>30.1</td>
<td>535:1</td>
<td>29.8, 25.5</td>
</tr>
<tr>
<td>295:1</td>
<td>33.8, 29.5, 26.4</td>
<td>710:1</td>
<td>30.2, 26.6</td>
<td>1045:1</td>
<td>30.0, 27.4</td>
</tr>
</tbody>
</table>

*Gradient gel electrophoresis was performed on interaction mixtures of vesicles with density subclasses of HDL. Gels were analyzed by computerized-densitometry as previously described (34).

**Molar ratio of phosphatidylcholine to HDL particle.

***No electrophoretic peaks were detected outside of the HDL migration range (34).
Figure 9. Histogram of all particle diameters for electrophoretic peaks observed outside of the HDL migration range. Gradient gel electrophoresis (PAA 4/30, 10 °C, 24 h, 125 V) was performed on all HDL incubation mixtures. Protein calibration standards were thyroglobulin (17.0 nm diameter), apoferritin (12.2 nm), lactate dehydrogenase (8.16 nm), and bovine serum albumin (7.1 nm). Gels were stained for protein (236). Apparent particle diameters were determined for peaks outside of the HDL migration range by computer analyses of densitometric scans using migration data for protein calibration standards (34). The non-HDL peak distribution is tabulated as a percentage of the total number of peaks observed outside of the HDL migration range. Peaks are included within diameter intervals when the peak has an apparent diameter, d, such that $n \leq d < m$ -- n and m are the interval limits.
subclasses, as shown in Table III. The frequency distribution of particle sizes (from peaks in GGE patterns) observed outside of the normal HDL migration interval is shown as a histogram in Figure 9. Maxima in the histogram appear at particle size intervals of 26-27 nm and 30-31 nm. Occasionally, at higher incubation ratios, particles with sizes larger than 32 nm were observed (Table III and Fig. 9). In general, however, the data of Table III show that, for a specific HDL density subclass, the particle sizes of these large products remain relatively constant with increasing incubation weight ratios. On the other hand, the peak areas increase with higher vesicle concentrations (data not shown). Incubation of HDL density subclasses in the absence of vesicles did not result in the formation of products in the 24.0 to 31.0 nm diameter range.

C. EFFECT OF HDL-VESICLE INTERACTION ON HDL DIAMETER

Figure 10 shows the incubation-induced (6 h, 37 °C) change in diameters for the size subpopulations of HDL as a function of the initial molar ratio (PC:HDL) in the incubation mixture. These findings were obtained from separate incubations of the density subclasses with vesicles. For comparison, particle size ranges for subpopulations of HDL (34) are included in this Figure (dashed lines). Particle sizes of both HDL₃ subpopulations ((HDL₃ᵃ)gge and (HDL₃ᵇ)gge) increased
Figure 10. Particle diameters (at electrophoretic peaks) for HDL density subclasses after a 6 h incubation (37 °C). Particle diameters are shown for (HDL$_{3a}$)gge, ○; (HDL$_{3b}$)gge, ●; (HDL$_{2a}$)gge, ○; and (HDL$_{2b}$)gge, △ incubated for 6 h at various molar ratios (PC:HDL). The dashed lines indicate the particle diameter ranges for HDL subpopulations (34).
linearly with increasing molar ratio. At the highest molar ratio, the mean particle size of both HDL\(_3\) subpopulations shifted into the \((\text{HDL}_{2a})_{gge}\) size interval. In contrast, both \((\text{HDL}_{2a})_{gge}\) and \((\text{HDL}_{2b})_{gge}\) size subpopulations showed only small increases in particle size with increasing molar ratio. Consequently, their particle sizes remained within the size intervals initially used to define these subpopulations; that is, \((\text{HDL}_{2a})_{gge}\) (8.8-9.8 nm) and \((\text{HDL}_{2b})_{gge}\) (9.8-12.6 nm). Based on the changes in diameter observed over the molar ratio range of 0:1 to 295:1, the \((\text{HDL}_{3})_{gge}\) subpopulations appeared to be the most responsive to incubation with vesicles. After a 6 h incubation with the concentrations of PC used, none of the subpopulations of HDL appeared to reach a limiting particle size.

After separate incubations of density subclasses of HDL with increasing amounts of vesicles for 24 h (Fig. 11), further increases in the diameters of all HDL size subpopulations were observed. At a molar ratio of 295:1, the diameters of the \((\text{HDL}_{3})_{gge}\) subpopulations appeared to approach limiting values of approximately 9.4 nm \((\text{HDL}_{3b})_{gge}\) and 10.2 nm \((\text{HDL}_{3a})_{gge}\). The size of the \((\text{HDL}_{3a})_{gge}\) subpopulation at the 295:1 molar ratio closely approximated the size of the \((\text{HDL}_{2a})_{gge}\) subpopulation at the same molar ratio. The \((\text{HDL}_{3b})_{gge}\) subpopulation shifted into the \((\text{HDL}_{2a})_{gge}\) size interval at molar ratios greater than 150:1. The \((\text{HDL}_{2a})_{gge}\) subpopulation
Figure 11. Particle diameters (at electrophoretic peaks) for HDL subclasses after a 24 h incubation (37 °C). Particle diameters are shown for (HDL$_3$)$_{gge}$, ○; (HDL$_3$)$_{bge}$, ●; (HDL$_2$)$_{a}$gge, O; and (HDL$_2$)$_{b}$gge, △ incubated for 24 h at various mole ratios (PC:HDL). The dashed lines indicate the particle diameter ranges for HDL subpopulations (34).
approached a limiting particle size of approximately 10.4 nm at a 710:1 (PC:HDL) molar ratio. At molar ratios above 345:1, the \( (\text{HDL}_{2a})_{gge} \) subpopulation entered into the \( (\text{HDL}_{2b})_{gge} \) size range, but it did not attain the particle size of the \( (\text{HDL}_{2b})_{gge} \) subpopulation used in this study. Above a molar ratio of 290:1, the \( (\text{HDL}_{2b})_{gge} \) subpopulation gradually increased in particle diameter and approached a limiting particle size of approximately 12.2 nm at an incubation ratio of 1045:1. At all molar ratios investigated, the \( (\text{HDL}_{2b})_{gge} \) subpopulation stayed within the previously-defined \( (\text{HDL}_{2b})_{gge} \) size interval.

The data for the \( (\text{HDL}_{2b})_{gge} \) subpopulation at the 0:1 molar ratio suggest some increase in particle diameter during incubation of this subpopulation alone. The apparent increase (0 h vs. 24 h) in particle size of the \( (\text{HDL}_{2b})_{gge} \) subpopulation was 0.7 nm. The changes observed for \( (\text{HDL}_{3})_{gge} \) and \( (\text{HDL}_{2a})_{gge} \) subpopulations, when these were separately incubated without vesicles, were within the range of precision (±0.1 nm) for the GGE-based measurements. Thus, the diameter of the \( (\text{HDL}_{2b})_{gge} \) subpopulation appears to be influenced to a greater extent than the diameters for \( (\text{HDL}_{3})_{gge} \) and \( (\text{HDL}_{2a})_{gge} \) subpopulations during a separate incubation at 37 °C.
Table IV. PHOSPHOLIPID:PROTEIN WEIGHT RATIOS OF THE DISCOIDAL AND LIPOPROTEIN PRODUCTS ISOLATED FROM HDL INTERACTION MIXTURES.†

<table>
<thead>
<tr>
<th>Incubation Ratio</th>
<th>HDL₃</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Discoidal Product</td>
<td>Lipoprotein Product</td>
</tr>
<tr>
<td>0:1</td>
<td>-</td>
<td>0.447:1</td>
</tr>
<tr>
<td>40:1</td>
<td>4.11:1</td>
<td>0.551:1</td>
</tr>
<tr>
<td>85:1</td>
<td>4.28:1</td>
<td>0.620:1</td>
</tr>
<tr>
<td>150:1</td>
<td>5.80:1</td>
<td>0.720:1</td>
</tr>
<tr>
<td>295:1</td>
<td>5.71:1</td>
<td>0.878:1</td>
</tr>
<tr>
<td>HDL₂a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Discoidal Product</td>
<td>Lipoprotein Product</td>
</tr>
<tr>
<td>0:1</td>
<td>-</td>
<td>0.562:1</td>
</tr>
<tr>
<td>90:1</td>
<td>6.75:1</td>
<td>0.670:1</td>
</tr>
<tr>
<td>175:1</td>
<td>6.18:1</td>
<td>0.798:1</td>
</tr>
<tr>
<td>345:1</td>
<td>6.10:1</td>
<td>0.945:1</td>
</tr>
<tr>
<td>710:1</td>
<td>7.05:1</td>
<td>1.18:1</td>
</tr>
<tr>
<td>HDL₂b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Discoidal Product</td>
<td>Lipoprotein Product</td>
</tr>
<tr>
<td>0:1</td>
<td>-</td>
<td>0.839:1</td>
</tr>
<tr>
<td>110:1</td>
<td>-</td>
<td>0.887:1</td>
</tr>
<tr>
<td>145:1</td>
<td>2.80:1</td>
<td>-</td>
</tr>
<tr>
<td>220:1</td>
<td>3.16:1</td>
<td>-</td>
</tr>
<tr>
<td>290:1</td>
<td>-</td>
<td>1.072:1</td>
</tr>
<tr>
<td>412:1</td>
<td>4.18:1</td>
<td>-</td>
</tr>
<tr>
<td>535:1</td>
<td>-</td>
<td>1.303:1</td>
</tr>
<tr>
<td>608:1</td>
<td>4.90:1</td>
<td>-</td>
</tr>
<tr>
<td>789:1</td>
<td>5.28:1</td>
<td>-</td>
</tr>
<tr>
<td>1045:1</td>
<td>-</td>
<td>1.655:1</td>
</tr>
</tbody>
</table>

Mean ± SD** 4.98:1 ± 0.90:1

†The discoidal and lipoprotein products were isolated in fractions 3-5 and 6-12 for HDL₃ mixtures; frs. 3-5 and frs. 7-12 for HDL₂ₐ mixtures; and frs. 5-9 (Fig. 15) and frs. 7-12 for HDL₂ₜ mixtures.

*Molar ratios (PC:HDL) for interaction mixtures.

**Mean and standard deviation of phospholipid to protein weight ratios for the discoidal products.
Figure 12. Fractionation of HDL$_3$ (HDL$_3$-PL, 0.705 mg/ml) incubation mixtures by equilibrium density gradient ultracentrifugation. HDL$_3$ were separately incubated (37°C, 6 h) with vesicles at various molar ratios (PC:HDL) and layered on a step-gradient as shown in Figure 3A. One ml fractions were sequentially removed after ultracentrifugation (Beckman SW-41 rotor, 36 h, 186,029 x g, 15°C). Protein (O) and phospholipid (X) concentrations were determined chemically (240,241). The fractionated incubation mixtures had the following molar ratios (PC:HDL): A) 0:1 B) 40:1 C) 85:1 D) 150:1 E) 295:1
D. FRACTIONATION AND CHARACTERIZATION OF INTERACTION PRODUCTS: HDL₃ INCUBATION MIXTURES

Each incubation mixture (6 h, 37 °C) was fractionated by equilibrium density gradient ultracentrifugation to investigate changes in lipoprotein chemical composition associated with the particle size changes. After ultracentrifugation, the distribution of phospholipid and apolipoprotein was determined throughout the preparative tube (Figs. 12-15). No sedimenting, lipid-free apolipoprotein was detected in the highest density fraction (fr. 12) for any of the incubation mixtures.

Density gradient ultracentrifugation of HDL₃, incubated for 6 h at 37 °C without vesicles, resulted in a peak of lipoprotein material primarily within the 1.113-1.154 g/ml density range (Fig. 12A). The mean phospholipid:protein weight ratio of this material (frs. 6-12) was 0.447:1 (Table IV). At the 40:1 molar ratio (PC:HDL) shown in Figure 12B, the distribution of HDL phospholipid and apolipoprotein shifted to lower density fractions; the phospholipid:protein weight ratio (frs. 6-12) was 0.551:1. In addition, an incubation product peak containing phospholipid and protein was distributed within fractions 3-5 corresponding to species with a mean hydrated density of 1.051 g/ml. The phospholipid:protein weight ratio for this product was 4.11:1 (Table IV).
At molar ratios of 85:1 and 150:1 (PC:HDL), the distribution of phospholipid and protein associated with the HDL₃ (frs. 6-12) progressively shifted further towards lower density fractions (Figs. 12C,D); the respective phospholipid to protein weight ratios were 0.620:1 and 0.720:1 (Table IV). At the 150:1 molar ratio (PC:HDL), the HDL₃ peak corresponded to species with mean hydrated densities of 1.113 g/ml. The product, distributed in fractions 3-5, progressively increased in concentration with increasing incubation ratios. The respective phospholipid:protein weight ratios of fraction 4 for mixtures at the 85:1 and 150:1 molar ratios were 4.28:1 and 5.80:1 (Table IV).

At a 295:1 (PC:HDL) molar ratio, there was no further decrease in mean hydrated density of the HDL₃; the density remained at 1.113 g/ml. The mean phospholipid to protein weight ratio of this product (frs. 6-12) was 0.878:1 (Table IV). The concentration of phospholipid and protein in fractions 3-5 increased over that observed for the 150:1 molar ratio. The phospholipid:protein weight ratio for this product was 5.71:1 (Table IV). The mean hydrated density for this product remained at 1.051 g/ml. At this molar ratio, some phospholipid and protein were also isolated, apparently in complex form, in fractions 1 and 2. These are the fractions in which vesicles are isolated when ultracentrifuged alone. For the 295:1 molar ratio, the complex of phospholipid and protein in fraction
1 was markedly enriched in phospholipid. The phospholipid:protein weight ratio was 21.0:1 for fraction 1.

To examine the morphology of the phospholipid-protein complex in fractions 1 and 2, aliquots of each were combined and then examined by electron microscopy using negative staining. A representative electron micrograph (Fig. 16A) shows liposomal structures. Thus, these fractions contain liposomal phospholipid with some associated apolipoprotein.

Electron microscopy was also performed on aliquots from fractions 3-5 isolated from all incubation mixtures containing vesicles. Particles in these samples consisted of discoidal complexes which stacked in rouleaux upon negative staining (Fig 16B). The major axis of these complexes was in the range of 25.0-30.3 nm, and a minor axis was 5.4 ± 1.2 (mean ± SD). The major axis based on EM was in good agreement with particle sizes outside of the HDL migration range based on GGE.

To determine the morphology of the HDL3 product isolated in fractions 6-9, separate aliquots of fractions 7 and 8 from each incubation mixture were examined by electron microscopy. Micrographs of native HDL3 and pooled aliquots of fractions 7 and 8 from the mixture at a 295:1 (PC:HDL) molar ratio are shown in Figure 18. Figure 18B shows spherical particles and is representative of micrographs of particles isolated in fractions 7 and 8.
comparable spheroidal particle shape is observed for native HDL₃ (Fig. 18A). Gradient gel electrophoresis of particles in fractions 7 and 8 for all mixtures showed their mean diameters to be identical to those previously determined during analysis of the respective unfraccionated total incubation mixtures (Fig. 10). Thus, in addition to liposomal and discoidal products, a phospholipid-enriched HDL₃ species resulted during incubation of the HDL₃ subclass with PC vesicles. These altered HDL₃ species exhibited an increased diameter and a decreased density with increasing molar ratios (PC:HDL).

Polyacrylamide gel electrophoresis, as described by Kane (237), was used to identify the apolipoproteins associated with the liposomal product in fraction 1 and with the discoidal product in fraction 4. Only apoA-I was obtained for the liposomal product from incubation mixtures containing HDL₃ (Fig. 17A, gel 1). Electrophoresis of the apolipoproteins from the discoidal product predominantly revealed (Fig. 17A, gel 2) apoA-I, but minor amounts of apoA-II were also observed. The ratio of apoA-I:apoA-II, obtained by densitometry, for the discoidal product was 4.5-fold greater than that determined for the native HDL₃ (Fig. 17A, gel 3,4). (Calibration curves of protein banding area, as determined by densitometry, versus the mass of protein applied were used to estimate the apoA-I to apoA-II ratio for the discoidal product.) The estimated apoA-I to apoA-II molar ratio for the discoidal
Figure 13. Fractionation of HDL2a (HDL2a-PL, 0.385 mg/ml) incubation mixtures by equilibrium density gradient ultracentrifugation. HDL2a were separately incubated (37 °C, 6 h) with vesicles and layered on a step-gradient as shown in Figure 3B. One ml fractions were sequentially removed after ultracentrifugation (Beckman SW-41 rotor, 36 h, 186,029 x g, 15 °C). Protein (O) and phospholipid (X) concentrations were determined by chemical methods (240,241). The fractionated incubation mixtures had the following molar ratios (PC:HDL): A) 0:1 B) 90:1 C) 175:1 D) 345:1 E) 710:1.
complex was 8:1. Thus, the protein moiety of the discoidal product consists predominantly of apoA-I.

E. FRACTIONATION AND CHARACTERIZATION OF INTERACTION PRODUCTS: HDL$_{2a}$ INCUBATION MIXTURES

Density gradient ultracentrifugation of HDL$_{2a}$, incubated for 6 h at 37 °C without vesicles, resulted in a peak of lipoprotein material primarily within the 1.091-1.116 g/ml density range (Fig. 13A). The mean phospholipid:protein weight ratio of this material (frs. 6-12) was 0.562:1 (PC:HDL). At a 90:1 molar ratio, there was no appreciable decrease in the HDL$_{2a}$ mean hydrated density; the mean phospholipid to protein weight ratio for this product (frs. 6-12) was 0.670:1 (Table IV). A separate phospholipid-protein complex species, with a mean hydrated density of approximately 1.056 g/ml, was isolated in fractions 3-5. The phospholipid:protein weight ratio for this product was 6.75:1 (Table IV).

For the incubation mixture at a 175:1 (PC:HDL) molar ratio (Fig. 13C), the HDL$_{2a}$ distribution shifted to lower density fractions. The mean phospholipid to protein weight ratio for this product (frs. 6-12) was 0.798:1. The concentrations of phospholipid and protein in fractions 3-6 increased over those obtained for the 90:1 mixture. The mean phospholipid to protein weight ratio of a pool of fractions 3 and 4 was 6.18:1 (Table IV).
The ultracentrifugal distribution of phospholipid and protein, for the 345:1 molar ratio mixture (Fig. 13D), indicated a further shift of the HDL$_{2a}$ to lower density fractions, with a peak at about 1.091 g/ml. The mean phospholipid to protein weight ratio of this product (frs. 6-12) was 0.945:1. There was also an increase in concentration of the interaction product isolated in fractions 3-5, without any appreciable change in its mean hydrated density. The average phospholipid:protein weight ratio for the product in fractions 3 and 4 was 6.10:1 (Table IV). In addition, the amount of material appearing in fractions 1 and 2 increased at this molar ratio compared with the 175:1 mixture. The mean phospholipid:protein weight ratio for the material in fraction 1 was 20.2:1.

For the 710:1 (PC:HDL) mixture, the HDL$_{2a}$ lipoprotein distribution shifted primarily into the 1.082-1.091 g/ml density fractions (Fig. 13E) and had a mean phospholipid to protein weight ratio of 1.183:1. The product in fractions 3-5 increased in both phospholipid and protein compared to the same fractions in the 345:1 mixture. The average phospholipid:protein weight ratio for the product in fractions 3 and 4 was 7.05:1. The level of material in fractions 1 and 2 also increased at the 710:1 mixture compared with those observed at the lower molar ratios. The mean phospholipid:protein weight ratio for the material in fraction 1 was 20.1:1.

Electron microscopy using negative staining was
performed on selected ultracentrifugal fractions to examine the morphology of the phospholipid-protein complexes. Fractions 1 and 2 from the 710:1 molar ratio sample appeared as liposomal structures.

Electron microscopy of pooled aliquots of fractions 3 and 4, for all mixtures containing vesicles, revealed the presence of discoidal complexes arranged in rouleaux. The major axis of these discs was in the range of 23.5-33.7 nm, while the minor axis was $5.7 \pm 1.1$ (mean $\pm$ SD). The major axis of this product compared favorably with particle sizes obtained by GGE for incubation products migrating outside of the HDL range.

Aliquots of fractions 9 and 10, from separate incubation mixtures with molar ratios ranging from 90:1 to 345:1 (PC:HDL), contained spherical particles that were similar to the lipoprotein particles in fractions 9 and 10 of the 0:1 mixture. Particles in fractions 8 and 9 of the 710:1 incubation mixture were also spherical in shape. GGE of the ultracentrifugal fractions subjected to EM showed the presence of particles with diameters identical to those observed during electrophoresis of the total incubation mixtures (Fig. 10). Thus, in addition to liposomal and discoidal products, a phospholipid-enriched HDL$_{2a}$ product resulted during incubation of the HDL$_{2a}$ density subclass with PC vesicles. For this latter product, the diameter increased and the density decreased with increasing incubation ratios (PC:HDL).
Figure 14. Fractionation of HDL$_{2b}$ (HDL$_{2b}$-PL, 0.859 mg/ml, HDL$_{2b}$-PL) incubation mixtures by equilibrium density gradient ultracentrifugation. HDL$_{2b}$ were separately incubated (37 °C, 6 h) with vesicles, layered on a step-gradient as shown in Figure 3C. One ml fractions were sequentially removed after ultracentrifugation (Beckman SW-41 rotor, 36 h, 186,029 x g, 15 °C). Protein (O) and phospholipid (X) concentrations were determined by chemical methods (240,241). The fractionated incubation mixtures had the following molar ratios (PC:HDL): A) 0:1 B) 110:1 C) 290:1 D) 535:1 E) 1045:1.
Figure 15. Fractionation of HDL₂b (HDL₂b-PL, 0.625 mg/ml) incubation mixtures by equilibrium density gradient ultracentrifugation. HDL₂b subclasses were separately incubated (37 °C, 6 h) with vesicles layered on a step-gradient as shown in Figure 3D. One ml fractions were sequentially removed after ultracentrifugation (Beckman SW-41 rotor, 36 h, 186,029 x g, 15 °C). Protein (O) and phospholipid (X) concentrations were determined by chemical methods (240,241). The fractionated incubation mixtures had the following molar ratios (PC:HDL): A) 0:1 B) 145:1 C) 220:1 D) 410:1 E) 610:1.
Polyacrylamide gel electrophoresis (237) was performed to identify the apolipoproteins associated with the various incubation products. As shown in Figure 17B (gel 1), apoA-I was the only protein detected in the liposomal product (fraction 1). The presence of minor amounts of other apolipoproteins could not be excluded. For the discoidal product (fractions 3 and 4; 20 µg/m of protein applied), apoA-I was the predominant component, although minor amounts of apoA-II were also observed (Fig. 17B; gel 2).

F. FRACTIONATION AND CHARACTERIZATION OF INTERACTION PRODUCTS: HDL\textsubscript{2b} INCUBATION MIXTURES

The initial fractionation scheme for HDL\textsubscript{2b} proved to be inadequate for resolution of liposomal and discoidal products banding in fractions 1-4 (Fig. 14). A modified density gradient provided excellent separation of these low density products (Fig. 15). This gradient, however, did not allow examination of changes in hydrated density of the HDL\textsubscript{2b}; consequently, both gradients were used for characterization of the incubation products. Density gradient ultracentrifugation of HDL\textsubscript{2b}, previously incubated alone, demonstrated a distribution of phospholipid and apolipoprotein components over the range of densities from 1.077 to 1.092 g/ml. The mean hydrated density of the HDL\textsubscript{2b} was approximately 1.084 g/ml (Fig.
The mean phospholipid:protein weight ratio of this material (frs. 5-12) was 0.839:1. Fractionation of mixtures at molar ratios from 110:1 to 1045:1 showed a progressive decrease in HDL$_{2b}$ hydrated density to a value of approximately 1.070 g/ml. The data of Figure 14 for fractions 5-12, containing the HDL$_{2b}$ at molar ratios of 110:1, 290:1, 535:1, and 1045:1, demonstrate mean phospholipid to protein ratios of 0.887:1, 1.072:1, 1.303:1, and 1.655:1 (Table IV), respectively.

Density gradient ultracentrifugation (Fig. 14B) of the mixture at a 110:1 molar ratio showed a product in the 1.050-1.054 g/ml density range. A product with a mean hydrated density of 1.051 g/ml is also observed in the ultracentrifugal fractionation shown in Figure 15B for a 145:1 (PC:HD) molar ratio. With increasing molar ratios (PC:HD), the product in fractions 5-9 (Fig. 15) showed an increase in phospholipid and apolipoprotein content but no change in peak hydrated density. The phospholipid:protein weight ratio for this product at incubation molar ratios of 145:1, 220:1, 410:1, 610:1, and 790:1 (PC:HD) was 2.80:1, 3.16:1, 4.18:1, 4.90:1, and 5.28:1, respectively.

For the mixture at a 220:1 molar ratio (Fig. 15C), material in fraction 1 was also observed. The level of phospholipid and protein in fractions 1-3 progressively increased with increasing molar ratio of PC:HD in the incubation mixture. The phospholipid:protein weight ratio of this product for 220:1, 412:1, 610:1, and 790:1 (PC:HD)
molar ratios was 5:1, 7:1, 8:1, and 12:1, respectively.

In the same manner as with the other HDL density subclasses, electron microscopy was used to characterize the morphology of the fractionated phospholipid-protein complexes. Fractions 1-3 (Fig. 15) contained liposomal structures. A representative electron micrograph of this product is shown in Figure 16A. Aliquots of fractions 5-8 (Fig. 15) were pooled and contained discoidal complexes which were stacked in rouleaux when examined by electron microscopy using negative staining. A representative electron micrograph of this product is shown in Figure 16B. Electron microscopy of fraction 9 (Fig. 14B) showed spherical particles (Fig. 18D) similar in size to those observed for native HDL\(_{2b}\) (Fig. 18C). Fraction 7 isolated from the 535:1 and 1045:1 (PC:HDL) incubation mixtures (Figs. 14D,E) also contained spherical lipoprotein particles when examined by electron microscopy (data not shown). For each of the incubation mixtures shown in Figure 14, GGE of the HDL\(_{2b}\)-containing fractions resulted in patterns with peaks in the HDL migration range. The diameters of the corresponding particles were identical to those obtained in the HDL migration range after electrophoresis of the respective total incubation mixtures (Fig. 10). Thus, HDL\(_{2b}\) forms liposomal, discoidal, and phospholipid-enriched HDL\(_{2b}\) products.

Polyacrylamide gel electrophoresis of selected fractions was performed to identify the associated
apolipoprotein components. For the liposomal product (fraction 1, Fig. 15), apoA-I was the predominant component, but minor amounts of the C apolipoproteins were also observed (Fig. 17C, gel 1). The discoidal product isolated in fractions 7 of Figure 15 (25 μgm of protein applied) only had apoA-I (Fig. 17C, gel 2). The presence of minor amounts of other apolipoproteins could not be excluded.

In summary, incubation of all HDL density subclasses (HDL₃, HDL₂a, and HDL₂b) resulted in the formation of three major products: liposomes with associated apolipoproteins, discoidal complexes of phospholipids and apolipoproteins, and lipoproteins with an altered chemical composition. The density of the lipoprotein product decreased progressively with increasing molar ratio (PC:HDL) of the incubation mixture. This physical-chemical change, as well as an increase in particle diameter, was associated with an increase in the phospholipid:protein weight ratio for this product. Specific changes in lipoprotein chemical composition associated with the increase in the phospholipid:protein ratio will be discussed subsequently.

Formation of discoidal complexes of phospholipid and apolipoprotein with a mean hydrated density in the range of 1.051-1.056 g/ml occurred in all interaction mixtures. The density for this product did not change appreciably with increasing incubation molar ratio. The
phospholipid:protein weight ratio of this complex, for a particular HDL density subclass, remained relatively constant. The average values for the phospholipid:protein weight ratios of the discoidal product obtained from HDL₃, HDL₂ᵃ, and HDL₂ᵇ mixtures were 4.89:1 ± 0.9:1, 6.52:1 ± 0.46:1, and 4.06:1 ± 1.07:1 (mean ± SD), respectively. Electron microscopy demonstrated that the major axis for these complexes was in the range of about 23-34 nm. Therefore, the peaks observed by GGE (Figs. 6B, 7B, and 8B) in this size range were probably due to the discoidal product. The progressive increase in staining intensity observed for this product in the gradient gels with increasing incubation ratios (PC:HDL) was consistent with the progressive increase in protein content of the ultracentrifugal fractions containing this product (Figs. 12-15). The predominant apolipoprotein associated with this product was apoA-I although small amounts of apoA-II were also noted.

At higher incubation molar ratios, a liposomal product was also observed that had some associated apolipoprotein. The phospholipid:protein weight ratio for this product was generally about 20:1. For HDL₃ and HDL₂ᵃ density subclasses, only apoA-I was detected in this product. However, apoA-II, and the C apolipoproteins also were noted in the liposomal product formed during HDL₂ᵇ incubations.
Figure 16. Electron micrographs of liposomal and discoidal products. Samples were dialyzed in a solution of 0.126 M ammonium acetate and 0.003 M ammonium carbonate (pH 7.4) and negatively stained with 2% sodium phosphotungstate as described in the materials and methods section. The bar marker in each panel represents 100 nm.
A. Representative liposomal product isolated in fraction 1 (Fig. 12).
B. Representative discoidal product isolated in fraction 4 (Fig. 12).
Figure 17. Electrophoresis of apolipoproteins of density subclasses of HDL and of incubation products. After delipidation (229), electrophoresis was performed using a discontinuous buffer system (238). Gels were stained for protein with a mixture of perchloric acid and Coomassie G-250 (236).

A. Gel 1, liposomal product (Fig. 12, fr. 1, 8 ugm protein applied) from HDL₃ incubation mixtures; Gel 2, discoidal product (Fig. 12, fr. 4, 36 ugm applied) from HDL₃ incubation mixtures; Gel 3, native HDL₃ (66 ugm applied); Gel 4, native HDL₃ (165 ugm applied).

B. Gel 1, liposomal product (Fig. 13, fr. 1, 15 ugm applied) from HDL₂ₐ incubation mixtures; Gel 2, discoidal product (Fig. 13, fr. 4, 20 ugm applied) from HDL₂ₐ incubation mixtures; Gel 3, native HDL₂ₐ (50 ugm applied); Gel 4, native HDL₂ₐ (130 ugm applied).

C. Gel 1, liposomal product (Fig. 15, fr. 1, 15 ugm applied); Gel 2, discoidal product (Fig. 15, fr. 7, 25 ugm applied); Gel 3, native HDL₂₉ (40 ugm applied); Gel 4, native HDL₂₉ (100 ugm applied).
Figure 18. Electron micrographs of the native HDL and the lipoprotein product. HDL3 (fractions 7,8, Fig. 12) and HDL2B (Fractions 7,8 of Fig. 14) were prepared as described in the legend to Figure 16 and were examined using 2% sodium phosphotungstate as a negative stain.


G. DISTRIBUTION OF PHOSPHOLIPID AND PROTEIN AMONG INCUBATION PRODUCTS

The results presented in Figures 12-15 were used to determine compositional changes in the incubated HDL density subclasses and to quantitate the relative distribution of apolipoprotein and phospholipid components among the three major incubation products (liposomal, discoidal, and lipoprotein products). For incubation mixtures containing the HDL$_3$ or HDL$_{2a}$ density subclasses, the ultracentrifugal fractions used in the compositional analysis were fraction 1 (the liposomal product), fractions 2-5 (the discoidal product), and fractions 6-12 (the lipoprotein product). For HDL$_{2b}$, data from two separate ultracentrifugal fractionations (Figs. 14 and 15) were used to obtain information on the three incubation products.

The compositional data indicated an increased phospholipid and a decreased apolipoprotein content in the lipoprotein product when compared with the native HDL density subclass. These results are consistent with an uptake of PC by the HDL, coupled with a release of some of its apolipoprotein.

Phospholipid uptake by each of the density subclasses of HDL increased with increasing molar ratio of PC:HDL (Fig. 19). At a ratio of 1045:1, uptake of phospholipid by HDL$_{2b}$ appeared to reach a limiting value of 48:1
Figure 19. Phospholipid uptake by HDL density subclasses. Estimates of the average molar uptake of phospholipid per HDL particle (PL:HDL) at various incubation ratios (PC:HDL,mol:mol) were determined from data in Figures 12-14. HDL₃, ●; HDL₂a, ○; HDL₂b, △.
(PL:HDL). In contrast, phospholipid uptake by HDL$_3$ and HDL$_{2a}$ had not reached a limiting value at the highest molar ratios examined (295:1 and 710:1, respectively). Uptake of phospholipid by HDL$_{2a}$ was greater than that by HDL$_{2b}$ at all molar ratios investigated. Comparison of uptake by the three subclasses at molar ratios below 300:1 shows that the density subclasses of HDL can be ranked in decreasing order of uptake as HDL$_{2a} >$ HDL$_3 >$ HDL$_{2b}$. The small change in particle diameter, noted by GGE, for HDL$_{2a}$ and HDL$_{2b}$ cannot be attributed to a lack of phospholipid uptake.

The data of Figures 12-15 also provide information on the apolipoprotein distribution among the various incubation products. Apolipoprotein that dissociated from the HDL density subclasses consisted almost exclusively of apoA-I. For example, the discoidal products from incubations of HDL$_3$ exhibited a molar ratio of apoA-I to apoA-II of about 8:1; native HDL$_3$ had an apoA-I:apoA-II molar ratio of 2:1 (9). The apparent number of apoA-I molecules dissociated per HDL particle increased with increasing molar ratios of PC to HDL (Fig. 20). Apolipoprotein dissociation from HDL$_3$ was slightly greater than that for the other two subclasses, up to an incubation ratio of 295:1. The HDL$_{2a}$ and HDL$_{2b}$ subclasses were comparable in apolipoprotein dissociation, up to a molar ratio of 350:1. At higher ratios, the apolipoprotein dissociated from HDL$_{2a}$ appeared to
Figure 20. Apolipoprotein dissociation from HDL subclasses. The data presented in Figures 12-14 were used to estimate the average number of apoA-I molecules dissociated per HDL particle (apoA-I:HDL) at various incubation ratios (PC:HDL, mol:mol). HDL_3, ⋄; HDL_{2a}, ○; HDL_{2b}, Δ.
approach a limiting value. For both HDL$_3$ and HDL$_{2b}$, the dissociation of apolipoprotein per particle continued to increase, up to the highest ratio examined, without approaching a limiting value. At the highest molar ratio (295:1) for HDL$_3$, apolipoprotein dissociation was about 0.75:1 (apoA-I:HDL). At the highest molar ratios (PC:HDL) for HDL$_{2a}$ and HDL$_{2b}$ (710:1 and 1045:1, respectively), the values for dissociated apolipoprotein were 1.2:1 and 2.1:1 apoA-I molecules per HDL particle, respectively. These values are maximal estimates for dissociation of this apolipoprotein since some apoA-II is also removed from the lipoprotein. As can be noted in Figures 12-15, the apolipoprotein dissociated from HDL subclasses was predominantly associated with the discoidal product. Less than 2, 5, and 13% of the total apolipoprotein was associated with the liposomal products in incubations involving HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$, respectively.

The distribution of phospholipid that was added to the incubation mixtures was determined. Greater than 65% of the "added" phospholipid was associated with both the lipoprotein (solid lines, Fig. 21) and discoidal (dashed lines, Fig. 21) products. The liposomal product only accounted for "added" phospholipid at high incubation ratios. The incorporation of "added" phospholipid into the lipoprotein product (expressed as a percentage of the total "added" phospholipid) was quite similar for HDL$_3$ and HDL$_{2a}$ over the range of incubation ratios from 0.90:1 to
Figure 21. Distribution of added phospholipid between the lipoprotein (solid line) and discoidal (dashed line) products. The data of Figures 12-15 were used to estimate the percent of the phospholipid, added to incubation mixtures, among the liposomal, discoidal, and lipoprotein products. The two predominant products, discoidal and lipoprotein, are shown. HDL$_3$, ○HDL$_{2a}$, △HDL$_{2b}$.
Both HDL$_3$ and HDL$_{2a}$ showed a progressive decrease in the percent of "added" phospholipid associated with the lipoprotein product with increasing incubation ratios; the maximum values observed for HDL$_3$ and HDL$_{2a}$ were 35% and 26%, respectively. In contrast, HDL$_{2b}$ appeared to take up a relatively constant percentage (5-10%) of "added" phospholipid at all ratios examined.

Over the range of incubation ratios investigated, between 60 and 75% of the "added" phospholipid was generally associated with the discoidal products. For interaction mixtures with HDL$_{2b}$, the discoidal products accounted for 68% of the "added" phospholipid at a molar ratio of 145:1 (PC:HDL). With increasing ratios, the percent of "added" phospholipid in discs decreased to 47% at a molar ratio of 790:1. The data for HDL$_3$ and HDL$_{2a}$ were similar over the molar ratio range of 90:1 to 295:1. A maximum value of about 75% was observed within the 150:1 to 175:1 range of molar ratios for both HDL$_3$ and HDL$_{2a}$.

Figure 21 clearly shows that the "added" phospholipid was predominantly involved in the formation of the discoidal product rather than the lipoprotein product.

The relationship between mass of phospholipid taken up and mass of apolipoprotein dissociated by HDL was evaluated by linear regression analysis for each subclass (Fig. 22). Correlation coefficients of 0.991, 0.993, and 0.934 were obtained for interactions involving HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$, respectively. The slopes of the regression lines
Figure 22. Linear regression analyses of data (Figures 12-14) for phospholipid uptake by and apolipoprotein dissociation from HDL density subclasses.
A. HDL\textsubscript{3}  B. HDL\textsubscript{2a}  C. HDL\textsubscript{2b}
for HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$ were 1.105, 0.934, and 0.476 (mg apolipoprotein per mg phospholipid), respectively. These values correspond to approximately 41, 35, and 18 phospholipid molecules taken up per one apoA-I dissociated. The lower slope for HDL$_{2b}$ probably reflects the observed saturation of phospholipid uptake by this lipoprotein subclass which occurs without a concomitant decrease in apolipoprotein dissociation. It is interesting to note that the slope (dashed line in Fig. 22) at low incubation ratios (110:1 and 290:1) is 0.949 (mg apolipoprotein per mg phospholipid), in good agreement with the values for HDL$_3$ and HDL$_{2a}$. This suggests that the relationship between the change in these components may be similar for all HDL subclasses except when phospholipid saturation is reached.

**H. INTERACTION OF TOTAL HDL WITH VESICLES**

To investigate vesicle-HDL interactions in native HDL mixtures, containing HDL$_{2b}$, HDL$_{2a}$, and HDL$_3$ density subclasses, incubations of vesicles with total HDL (HDL-PL, 1.37 mg/ml) were performed. The GGE data after a 24 h incubation (37°C) of a representative total HDL preparation with various amounts of vesicles (0:1, 0.60:1, 0.95:1, 1.95:1, 3.85:1; SUV-PC:HDL-PL, wt:wt) are shown in Figure 23. The changes in subpopulation diameters in the native mixture of total HDL were compared with those
Figure 23. Particle diameters of HDL size subpopulations for incubation mixtures of total HDL and vesicles. Total HDL (HDL-PL, 1.37 mg/ml) were incubated (37 °C) for 24 h with vesicles at weight ratios of 0:1, 0.57:1, 0.95:1, 1.96:1, and 3.83:1 (SUV-PC:HDL-PL). Gradient gel electrophoresis was performed and particle diameters were determined as described in the legend to Figure 9. (HDL$_{3b}$)gge, ● ; (HDL$_{3a}$)gge, ○ ; (HDL$_{2a}$)gge, ○ ; (HDL$_{2b}$)gge, △.
previously described for the isolated HDL subclasses (Fig. 11).

At weight ratios below 1:1, the particle diameters for (HDL\textsubscript{2a})\textsuperscript{gge} and (HDL\textsubscript{2b})\textsuperscript{gge} subpopulations remained at 9.3 and 10.7 nm, respectively. In contrast, there was a linear increase in (HDL\textsubscript{3a})\textsuperscript{gge} and (HDL\textsubscript{3b})\textsuperscript{gge} diameters with increasing PC up to a weight ratio of 1:1 (SUV-PC:HDLL-PL). At the 1:1 ratio, the peak for (HDL\textsubscript{3a})\textsuperscript{gge} overlapped the (HDL\textsubscript{2a})\textsuperscript{gge} peak. There was no resolution of the peaks at higher incubation ratios. These observations were consistent with data from incubations of isolated subclasses with vesicles (Fig. 11) which showed that (HDL\textsubscript{3a})\textsuperscript{gge} and (HDL\textsubscript{2a})\textsuperscript{gge} subpopulations have comparable diameters in incubation mixtures with molar ratios greater than 175:1 (PC:HDL).

At weight ratios above 1:1, the (HDL\textsubscript{2b})\textsuperscript{gge} subpopulation diameter increased and approached a limiting size when the ratio reached 3.85:1. The overlap peak, comprising (HDL\textsubscript{2a})\textsuperscript{gge} and (HDL\textsubscript{3a})\textsuperscript{gge} subpopulations, continued to increase with increasing levels of PC. The diameter of the (HDL\textsubscript{3b})\textsuperscript{gge} subpopulation also increased with increasing PC and approached a limiting size at the 3.8:1 weight ratio.

Analytical ultracentrifugation of the total HDL used in the above studies, together with three-component computer analysis of the sclieren pattern (24), gave HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b} plasma concentrations of 96,
107, and 126 mg/dl, respectively. Based on the chemical compositions of these density subclasses (24), the HDL₃, HDL₂a, and HDL₂b subclasses constitute 20%, 35%, and 45% of the total HDL phospholipid concentration, respectively, in this total HDL sample. Thus, a 1:1 weight ratio (SUV-PC:HDL-PL) for the total HDL corresponds to respective molar ratios (PC:HDL) for HDL₃, HDL₂a, and HDL₂b of 265:1, 355:1, and 475:1. At comparable weight ratios, the diameters of subpopulations in isolated density subclasses of HDL were consistently greater than those of the corresponding subpopulation in the total HDL mixture. Thus, none of the HDL subpopulations in a native mixture of HDL interact with vesicles as if they were independent of the presence of the other HDL subpopulations. These data indicate that all HDL subclasses compete for vesicle PC under these experimental conditions.
Electrophoresis was performed on 2-16% polyacrylamide gradient gels as described in the legend to Figure 9. LDL-PL concentration was 0.63 mg/ml in each mixture. Protein staining pattern for the electrophoretic gel is shown. Lane 1, nonincubated LDL; lanes 2-7 were incubated mixtures (37 °C, 6 h) with the following weight ratios (SUV-PC:LDL-PL): lane 2, 0:1; lane 3, 0.20:1; lane 4, 0.40:1; lane 5, 1.00:1; lane 6, 2.00:1; lane 7, 4.00:1. Lane 8 contains particle size calibration standards of carboxylated latex beads (major peak diameter, 38.0 nm; Dow Diagnostics) and of High Molecular Weight Standard Mix (see legend to Figure 9). Only the thyroglobulin band is shown.
Gel Top
Latex Beads (38.0 nm)
LDL
Thyroglobulin (17.0 nm)
I. CHARACTERIZATION OF LDL

Gradient gel electrophoretic patterns of unincubated LDL showed multiple bands when either protein or lipid stain was used (Fig. 24, lane 1). For the gradient gel shown in Figure 24 (lane 1), particle diameters of 25.6, 24.4, and 22.5 nm were obtained from peaks in the densitometric scan. The above particle diameters for size subpopulations of LDL fell within the range of values previously reported for normal subjects (29). No peaks that would indicate the presence of lipoprotein(a) or plasma proteins were detected by GGE of isolated LDL.

J. LDL-VESICLE INTERACTION STUDIES

For preliminary studies, incubation mixtures contained LDL at a constant concentration (LDL-PL, 0.63 mg/ml), similar to the levels found in normal plasma, and vesicles at incubation weight ratios ranging from 0.20:1 to 4.00:1 (SUV-PC:LDL-PL). These mixtures were examined by GGE in order to evaluate possible changes in lipoprotein particle size, as noted previously for HDL. Figure 24 shows an electrophoretic gel stained for protein. The pattern (lane 2) for LDL incubated alone (6 h, 37 °C) is the same as that for unincubated LDL (lane 1). Separate incubations (6
Figure 25. Staining intensity of LDL bands in electrophoretic gels. At each incubation weight ratio, the staining intensity was determined from the densitometric scan area of the LDL peaks in Fig. 24A. These data are shown as a percentage of the densitometric scan area for control LDL.
h, 37 °C) of LDL with increasing amounts of vesicles for material contained within the LDL migration range (Fig. 25). (Banding area is defined as the area under densitometric peaks.) The banding area decreased to 64% of the control value for LDL at a 2.00:1 weight ratio and decreased to 36% at a 4.00:1 ratio (SUV-PC:LDL-PL). In contrast to HDL, there was no apparent shift of LDL peaks to smaller migration positions.

Concomitant with the decrease in area for LDL, a band of material progressively increased in staining intensity near the top of the gel. This material was not within the migration range for LDL and had apparent dimensions greater than 50 nm. There were no incubation products banding between LDL and the material near the top of the gel. Similar findings were obtained when electrophoretic gels of incubation mixtures were stained for lipid. When stained for protein or lipid, neither incubated nor unincubated vesicles contributed to the staining near the top of the gel. Thus, the GGE data indicated a shift of LDL protein and lipid into a gel region corresponding to particles with dimensions greater than 50 nm.
Figure 26. Fractionation of LDL incubation mixtures by velocity ultracentrifugation. One ml of incubation mixture was layered on a step density gradient of 1.019 g/ml (5 ml) and 1.032 g/ml (6 ml). After ultracentrifugation (Beckman SW-41 rotor, 12 h, 186,029 x g, 17 °C), one ml fractions were sequentially removed. Protein (●) and phospholipid (○) were determined by chemical methods (240,241); total cholesterol (×) was determined by an enzymatic assay.

A. LDL and vesicles incubated separately.
B. Vesicle-LDL incubation mixture (LDL-PL, 0.55 mg/ml; SUV-PC, 1.10 mg/ml).
K. FRACTIONATION AND CHARACTERIZATION OF LDL-VESICLE INTERACTION PRODUCTS

Velocity ultracentrifugation on a step density gradient was used to isolate the large products (dimension >50 nm) observed by GGE from a 2.00:1 (SUV-PC:LDL-PL, wt:wt; LDL-PL, 0.550 mg/ml) incubation mixture (6h, 37 °C). This mixture was shown by GGE to have both LDL and the large products. Control LDL and vesicles were also fractionated by velocity ultracentrifugation. Chemical analyses were performed on all ultracentrifugal fractions (Fig. 26).

After ultracentrifugation, control vesicles were found predominantly in fraction 1; control incubated LDL sedimented into fractions 3-6 with the peak of the distribution in fraction 5 (Fig. 26A). After ultracentrifugation, the LDL-vesicle interaction mixture showed an apparent trimodal distribution of phospholipid and protein (Fig. 26B). Fraction 2 contained a predominant peak in the phospholipid distribution. Fractions 1 and 2 also contained small amounts of protein (about 6% of total protein) and cholesterol (about 20% of total cholesterol). Fraction 5 contained a predominant peak in the protein distribution and consisted of material with a cholesterol:protein weight ratio and phospholipid:protein ratio identical to native LDL. Fraction 7 contained peaks in both the phospholipid and protein distributions and was
Figure 27. Electron micrographs of vesicles, LDL, and isolated turbid product. Samples were prepared for electron microscopy as described in the legend to Figure 16.

A. Control PC vesicles. These tend to collapse and form rouleaux during negative staining (mean ± SD dimensions, 27.3 ± 3.3 nm x 11.9 ± 2.0 nm). Occasionally vesicles trap the negative stain (arrows).

B. Control LDL. These consist mainly of free-standing round particles (26.1 ± 3.1 nm) which are polygonal when closely packed.

C. Turbid product formed during a 24 h incubation and isolated in fraction 7 (Fig. 26). This consists of large irregular aggregates of particles (29.2 ± 5.7 nm) having apparent dimensions slightly larger than the native LDL and vesicles. Bar marker represents 100 nm and is the same for all panels.
turbid. Material in fraction 7 had a cholesterol:protein weight ratio (1.5:1) similar to that of control LDL (1.4:1) but a phospholipid:protein weight ratio (2.7:1) over threefold that of LDL (0.8:1).

Two possible explanations for the mass distribution observed by GGE and ultracentrifugation include: (1) LDL take up vesicle phospholipid to form particles with diameters greater than about 50 nm or (2) LDL and vesicles, essentially retaining their original diameters, aggregate to form products with overall dimensions greater than 50 nm.

Electron microscopy was employed to characterize the turbid fraction (fraction 7) in an attempt to distinguish between the two explanations given above. Unincubated LDL (Fig. 27A) were spherical particles with a diameter of $26.1 \pm 3.1$ nm (mean ± SD). Unincubated vesicles (Fig. 27B) appeared as rouleaux of collapsed particles with a major axis of $27.3 \pm 3.3$ nm and a minor axis of $11.9 \pm 2.0$ nm. The turbid product isolated in fraction 7 (Fig. 27C) consisted of closely associated particles with an average dimension ($29.2 \pm 5.7$ nm) moderately larger than that for the starting LDL and vesicles. Collapsed vesicles also were occasionally observed within fraction 7. These findings indicate that the large (>50 nm) and phospholipid-enriched products (ultracentrifugal fraction 7) result primarily from aggregation of intact LDL with vesicles rather than from phospholipid incorporation by
LDL. The possibility cannot by excluded, however, that LDL may take up some PC without a significant change in particle diameter.

Based on the compositional data obtained for ultracentrifugal fractions and on the assumption that no phospholipid was taken up by LDL, the molar ratio for the LDL-vesicle aggregate would be 2:1 (LDL:vesicle). This estimated ratio also used a molecular weight for LDL and SUV of $2.3 \times 10^6$ (13) and $2.06 \times 10^6$ (231), respectively; the assumed phospholipid composition for LDL was 22% of the lipoprotein mass (13).

Interaction of LDL with vesicles in the absence or presence of an anti-oxidant (0.05% reduced glutathione) resulted in identical particle size and turbidity findings. Moreover, no small molecular weight fragments of apoB, indicative of oxidative degradation of the protein (245), were observed by SDS polyacrylamide gel electrophoresis. Thus, aggregation between lipoproteins and vesicles was not the result of oxidative changes in either reactant.

L. KINETICS AND MECHANISM OF LDL-VESICLE AGGREGATION

Aggregation of LDL with vesicles increased the solution turbidity. (Turbidity is 2.303 times the absorbance (246).) Thus, the time course of the increase in turbidity could be monitored spectrophotometrically and could be used to investigate the kinetics of aggregation.
Figure 28. Absorbance time course for LDL incubation mixtures. LDL (LDL-PL, 0.585 mg/ml) incubation (37 °C) mixtures at weight ratios (SUV-PC:LDL-PL) of 0.0:1 (●), 0.40:1 (○), 1.00:1 (■), 2.00:1 (□), and 4.00:1 (△) were monitored spectrophotometrically at 340 nm.
For Rayleigh scattering, the relationship, at low concentrations of scattering particles, between absorbance (A) and concentration (C) of light-scattering aggregation products is given by the following equation (246):

$$A = \frac{16\pi}{6.909}KCM$$

The term K is a constant that is related to the refractive index for the solvent ($n_0$), the specific refractive increment for the solute ($\Delta n/\Delta c$), Avogadro's number (N), and the wavelength of light in the medium ($\lambda$) by the following expression:

$$K = 2\pi^2 (n_0)^2 \left(\frac{n}{c}\right)^2 / (N) (1/\lambda)^4$$

Thus, at a fixed wavelength, an increase in the absorbance of an LDL-vesicle interaction mixture can result from an increase in either concentration or molecular weight of the light-scattering aggregate. Figure 28 shows typical absorbance data for incubation mixtures (37 °C) of LDL at a constant concentration (0.585 mg/ml) with various amounts of vesicles. At weight ratios from 0.40:1 to 4.00:1 (SUV-PC:LDL-PL), incubation mixtures approached limiting absorbances at 24 h that were proportional to the weight ratios.

Reaction kinetics were also obtained from absorbance data and were used to distinguish possible reaction mechanisms. In the case of LDL-vesicle aggregation, the reaction kinetics were analyzed based on three pathways:
Pathway 1: \[ A + B \xrightleftharpoons[k_{-1}][k_{1}] AB \]

Pathway 2: \[ 2A + B \xrightleftharpoons[k_{-2}][k_{2}] A_2B \]

Pathway 3: \[ A + B \xrightleftharpoons[k_{-3}][k_{3}] AB + A \xrightleftharpoons[k_{-4}][k_{4}] A_2B \]

In these reactions, \( A \) denotes either LDL or vesicles, and \( B \) denotes the reactant not represented by \( A \); \( AB \) and \( A_2B \) denote aggregation products. The three pathways shown were found to be sufficient for interpreting the kinetic data. Therefore, reaction pathways leading to aggregate stoichiometries other than 1:1, 1:2, and 2:1 (LDL:SUV,mol:mol) are not described.

For pathway 1, the kinetic expression for aggregate production can be written in a simplified form, at early reaction times when the concentration of \( AB \) is low, as

\[
(1) \quad \frac{d(AB)}{dt} = k_1 (A) (B)
\]

In expression 1 and all other kinetic expressions, the concentration of a reactant or product species, \( Z \), is written as \( (Z) \).

For pathway 2, the kinetic expression can be written, for early reaction times when the concentration of \( A_2B \) is low, as
For pathway 3, two limiting cases can be analyzed. First, assuming that $k_3 \gg k_4$, the rate-limiting step in $A_2B$ production would be aggregation of $AB$ with $A$. Since $AB$ is light-scattering, the production of $AB$ would be detected spectrophotometrically prior to the formation of $A_2B$. Thus, for absorbance measurements, the appropriate kinetic expression can be written for early reaction times, when the concentrations of $A$ and $B$ are practically constant and the concentration of $A_2B$ is negligible, as

\[
(3) \quad \frac{d(AB)}{dt} = k_3(A)(B)
\]

Second, assuming that $k_4 \gg k_3$, the rate-limiting step in $A_2B$ production would be the formation of $AB$ by aggregation of $A$ with $B$. At early reaction times, the concentration of $A_2B$ is low; and the concentrations of $A$ and $B$ are practically constant. In this case, the kinetic expression can be written as

\[
(4) \quad \frac{d(A_2B)}{dt} = k_3(A)(B)
\]

The overall reaction kinetics, therefore, would be third-order based on expression 2 and second-order based on expressions 1, 3, and 4.

The reaction order with respect to either LDL or
vesicles was evaluated by monitoring the rate of aggregate formation in mixtures having various concentrations of the reactant of interest (LDL or vesicles) and an elevated, hence relatively constant, concentration of the other reactant. As noted previously, absorbance is proportional to both the molecular weight and the concentration of light-scattering species. Assuming that only one major aggregation product (either AB or A₂B) is formed at early times, the absorbance would, therefore, be proportional only to aggregate concentration; and the initial slope of the absorbance data would be proportional to the rate of change of aggregate concentration. Reaction order can then be obtained by a plot of initial slope versus concentration of the reactant of interest; a linear plot indicates first-order kinetics. Higher-order kinetics can be determined from the slope of a plot of ln(reaction rate) versus ln(reactant concentration).

The Rayleigh equations are strictly applicable to light scattering by particles that have dimensions less than about \( \lambda/20 \) (246). For particles above this size, intramolecular interference reduces the scattering intensity, which is given by the expression \( P(\theta) = I_0/I \).

The terms \( I \) and \( I_0 \) are the scattering intensities due to Rayleigh-scattering particles and to particles that have a radius of gyration between \( \lambda/20 \) and \( \lambda/2 \), respectively. The value of \( P(\theta) \) is between 0 and 1 and is a function of scattering angle and the radius of gyration for the
Figure 29. Reaction rates (change in absorbance per hour) for LDL incubation mixtures.
A. The initial reaction rates were obtained for LDL (0.320 mg/ml) incubation mixtures at weight ratios of 0.77:1, 1.84:1, 3.68:1, and 7.36:1 (SUV-PC:LDL-PL).
B. The initial reaction rates were obtained for incubation mixtures that had a constant concentration of vesicles (SUV-PC, 18.116 mg/ml) and increasing amounts of LDL to give weight ratios of 114.66:1, 84.65:1, and 57.15:1 (SUV-PC:LDL-PL).
B

2nd order

Rate (A/h)

LDL-PL (mg/ml)

r=0.997
m=5.000
scattering particle relative to the wavelength of light. The radius of gyration for the LDL-vesicle aggregation product is approximately 25 nm, based on the GGE findings. This dimension is equivalent to about $\lambda/14$ at the wavelength used for spectrophotometry. Therefore, some intramolecular interference would be expected to occur. This effect, however, should not alter the interpretation of reaction rates based on the initial slopes of the absorbance data but would merely increase the absorbance values by a constant factor related to the radius of gyration for the aggregate.

The reaction order with respect to vesicles was determined from incubation mixtures containing LDL at a phospholipid concentration of 0.320 mg/ml and vesicles at a PC concentration of 0.245, 0.589, 1.177, and 2.356 mg/ml. The respective molar incubation ratios were estimated to be 9.7:1, 4.0:1, 2.0:1, and 1.0:1 (LDL:SUV). The initial slopes of the absorbance data versus SUV-PC concentration yielded a linear relationship with a slope of 0.080 Å/h per mg/ml of PC and a correlation coefficient of 0.998 (Fig. 29A).

The reaction order with respect to LDL was determined from incubation mixtures containing vesicles at a PC concentration of 18.116 mg/ml and LDL at phospholipid concentrations of 0.158, 0.214, and 0.317 mg/ml. The respective molar incubation ratios were estimated to be 20.0:1, 14.8:1, 10.0:1 (SUV:LDL). A plot of the
Figure 30. Time course of absorbance change of LDL incubation mixtures at various temperatures. The LDL (LDL-PL, 0.560 mg/ml) were incubated with vesicles (1.120 mg/ml) at temperatures of 5 (▲), 14 (△), 24 (■), 30 (□), and 37 °C (●, ○); incubation mixtures were monitored spectrophotometrically at 340 nm.
initial-slope data versus LDL concentration was linear with a slope of 5.0 \text{ A/h per mg/ml} of LDL phospholipid and correlation coefficient of 0.997 (Fig. 29B).

Second-order kinetics for reactants are shown as a dashed line in Figures 29A and 29B. These hypothetical curves include the contribution of \( A_2B \) molecular weight to the absorbance. Since the actual kinetics were first-order with respect to both LDL and vesicles, the overall reaction of LDL-vesicle aggregation was second-order. Based on the reaction kinetics for LDL and vesicles, the average value for the rate constant \( (k_1 \text{ or } k_3) \) was estimated to be 0.28 \( \text{ (A/h)} (\text{ml/mg})^2 \).

These findings rule out the direct formation of \( A_2B \) but are consistent with sequential formation of this product. In fact, the kinetic data would be consistent with a sequential formation of other aggregation products \( A_nB_m \) that go through an initial aggregate of \( AB \). The kinetic findings also are consistent with pathway 1, formation of aggregate \( AB \). Thus, the overall reaction kinetics support the initial aggregation of LDL with vesicles at a 1:1 (LDL:SUV) molar stoichiometry but cannot rule out subsequent addition of either LDL or vesicles to this product.

In an effort to estimate the activation energy for LDL-vesicle aggregation, absorbance data (Fig. 30) were obtained for mixtures \( (\text{LDL-PL, 0.56 mg/ml; SUV-PC, 1.12 mg/ml}) \) at 5, 14, 24, 30, and 37 \( ^\circ \text{C} \). The initial slopes
Figure 31. Reaction rates (change in absorbance per hour) of LDL incubation mixtures at various temperatures. The initial reaction rates are shown for 2.00:1 (SUV-PC:LDL-PL) weight ratio mixtures that were incubated at 5, 14, 24, 30, and 37 °C. The dashed line is an extrapolation of the data.
of the absorbance data were used as a measure of the initial reaction rates. The activation energy, however, could not be determined from the experimental findings because the Arrhenius plot of initial slope versus temperature did not yield a straight line. The respective initial rates obtained at incubation temperatures of 5, 14, 24, 30, and 37 °C were 0.149, 0.133, 0.115, 0.131, and 0.206 A/h. A plot of these data on linear axes is shown in Figure 31. Extrapolation of the initial rate data (dashed lines, Fig. 31) suggested a minimum rate at about 28 °C. When LDL isolated from other subjects were investigated, studies showed that the extrapolated minimum rate for each subject was in the temperature range of 21-28 °C. Additional experiments (data not shown) demonstrated that the slowest kinetics, indeed, occurred at the temperature estimated by extrapolation of the reaction rate data.

The absorbances attained at 24 h for the incubation mixtures at a 2.00:1 (SUV-PC:LDL-PL) weight ratio were a function of temperature; mixtures incubated at 5 and 14 °C had absorbances above those obtained at higher incubation temperatures (Fig. 30). These data were determined for mixtures at a specific weight ratio (2.00:1) and, therefore, indicated that either aggregate stoichiometry or concentration varied with temperature, the incubation mixtures had not reached equilibrium, or both explanations were valid. Although additional studies of longer incubation periods indicated a gradual increase in
absorbance after 24 h, limiting absorbances were reached at 72 h (data not shown). For all LDL samples investigated, the limiting absorbances were greater for mixtures incubated at 5 °C than at 14 °C; mixtures incubated at 14 °C had larger limiting absorbances than samples incubated at either 24, 30, or 37 °C. The variability among data for different LDL samples that have been incubated at 24, 30, and 37 °C precluded generalizations about their relative absorbances. The temperature-dependent variations in the final absorbances, therefore, suggested that either aggregate stoichiometry, concentration, or both varied with temperature.

M. EFFECTS OF TEMPERATURE ON LDL-VESICLE INTERACTIONS

After a 24 h incubation, mixtures at a 2.00:1 weight ratio (SUV-PC:LDL-PL), described above for incubation temperatures at 5, 14, 30, and 37 °C, were fractionated by velocity ultracentrifugation. Chemical characterization of the ultracentrifugal fractions was performed to investigate whether absorption differences were due to temperature-dependent variations of aggregate concentration, aggregate stoichiometry, or both factors. Vesicles used in these incubation mixtures contained inulin in order to estimate phospholipid uptake by aggregated LDL. These estimates will be described in the Discussion section.
Figure 32. Fractionation of LDL incubation mixtures by velocity ultracentrifugation. LDL (LDL-PL, 0.560 mg/ml) were incubated (5, 14, 30, and 37 °C) for 24 h with vesicles at a 2.00:1 weight ratio (SUV-PC:LDL-PL). These mixtures were prepared by layering 1 ml of the sample above a linear salt gradient (1.019-1.030 g/ml). After ultracentrifugation (Beckman SW-41 rotor, 12 h, 186,029 x g, 17 °C), one ml fractions were sequentially removed from the ultracentrifuge tube. Protein, ● (240) and phospholipid, ○ (241) concentrations were determined by chemical methods. A) Vesicles incubated alone. B) LDL incubated alone. C) 37 °C incubation mixture. D) 30 °C incubation mixture. E) 14 °C incubation mixture. F) 5 °C incubation mixture.
After a 12 h ultracentrifugation period, one ml fractions were sequentially removed from the ultracentrifuge tubes and analyzed for protein and phospholipid. As described previously, initial fractionation studies of LDL-vesicle incubation mixtures utilized velocity ultracentrifugation with a step density gradient (Fig. 26). The studies that are presented here employed velocity ultracentrifugation with a linear density gradient. This technique was better than the step-gradient procedure for separation of the three components of incubation mixtures: vesicles, LDL, and aggregation products.

Incubated vesicles were isolated in ultracentrifugal fractions 1 and 2. Incubated LDL were isolated within fractions 3-6 (Figs. 32 A,B). For incubation mixtures containing both LDL and vesicles, major peaks of phospholipid and protein were distributed within fractions 7-12; these fractions contained the aggregation products. Separate pools of the above fractions were used for chemical characterization of LDL and turbid aggregation products.

After ultracentrifugation of the 5 and 14 °C incubation samples, a trimodal distribution of phospholipid was observed (Fig. 32 E,F). Peaks were observed corresponding to vesicles, LDL, and aggregation products. The 30 and 37 °C incubation mixtures showed a bimodal distribution of phospholipid with peaks in fractions...
Table V. CHARACTERIZATION OF THE LDL-VESICLE INTERACTION MIXTURE AND ISOLATED PRODUCTS.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>LDL Product Weight Ratio* (PL:Protein)</th>
<th>Aggregation Product Weight Ratio** (PL:Protein)</th>
<th>Molar Ratio*** Model 1</th>
<th>Molar Ratio Model 2</th>
<th>Inulin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1.31:1</td>
<td>3.69:1</td>
<td>1.9:1</td>
<td>4.0:1</td>
<td>57</td>
</tr>
<tr>
<td>30</td>
<td>1.23:1</td>
<td>4.12:1</td>
<td>1.5:1</td>
<td>3.0:1</td>
<td>52</td>
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<tr>
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<td>1.04:1</td>
<td>3.46:1</td>
<td>1.2:1</td>
<td>4.0:1</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>0.99:1</td>
<td>2.85:1</td>
<td>2.2:1</td>
<td>6.3:1</td>
<td>51</td>
</tr>
</tbody>
</table>

*Phospholipid:protein weight ratios for the non-aggregated LDL isolated in fractions 3-6 (Fig. 32).

**Phospholipid:protein weight ratios for the aggregation product isolated in fractions 7-12 (Fig. 32).

***Values are the estimated molar stoichiometries (SUV:LDL) for the aggregation products isolated in fractions 7-12 (Fig. 32). The two models used for the estimation are discussed in the text.
corresponding to LDL and aggregation products. A shoulder (fraction 2) on the peak for LDL suggested the presence of some vesicles (Figs. 32 C, D). The protein distribution was bimodal for all incubation mixtures. Apolipoprotein B was isolated in fractions containing LDL and aggregation products; no apolipoprotein was associated with the vesicles.

Based on the results shown in Fig. 32, the weight ratios (phospholipid:protein) for aggregation products (fractions 7-12) were obtained (Table V). For the aggregates formed during incubations at 5, 14, 30, and 37 °C, the weight ratios were 2.83:1, 3.43:1, 3.98:1, and 3.46:1 (phospholipid:protein), respectively. The weight ratio for control unincubated LDL was 1.00:1. Thus, ratios for the aggregation products were about 3-4 times that for the native lipoprotein.

The weight ratios for non-aggregated LDL (fractions 3-6) and the percentage of LDL involved in aggregation were also obtained from the chemical data (Fig. 32). Non-aggregated LDL incubated at 5, 14, 30, and 37 °C had respective ratios (Table V) of 0.99:1, 1.04:1, 1.23:1, and 1.31:1 (phospholipid:protein). Since the ratios for LDL incubated at 5 °C and for control LDL were similar, the lipoproteins apparently did not take up phospholipid at this temperature. Because apoB was not dissociated from LDL, the increase in phospholipid content of non-aggregated LDL was 4%, 23%, and 31%, respectively. Based on the
amount of apoB in ultracentrifugal fractions 7-12, aggregation products formed at 5, 14, 30, and 37 °C involved 61%, 57%, 48%, and 55% of the total LDL after a 24 h incubation.

In summary, the phospholipid to protein weight ratios for the aggregation products tended to increase with an increase in incubation temperature. The aggregates formed at 37 °C, however, were not consistent with this relationship between weight ratio and temperature; instead of an extrapolated ratio of 4.1:1, the aggregation products formed at 37 °C had a weight ratio of 3.46:1 (phospholipid:protein). The mixtures, except those incubated at 37 °C, also demonstrated a decrease in the percent of total LDL involved in aggregation with an increase in incubation temperature. Aggregation products formed at 37 °C involved 55% of the LDL rather than 44% of the LDL which would be estimated based on data extrapolation. Despite deviations at 37 °C from trends suggested by data obtained at the 5, 14, and 30 °C incubation temperatures, an inverse relationship between aggregate weight ratios (phospholipid:protein) and the percent of the total LDL that form aggregates was observed at all incubation temperatures. Linear regression analysis of these parameters gave a correlation coefficient of 0.942.

The observed temperature-dependence of the final absorbances for the incubation mixtures are consistent with
Figure 33. Time course of absorbance change of enzyme-treated LDL incubated with vesicles. LDL (0.554 mg/ml) were pre-treated with either trypsin (○), papain (●), or neuraminidase (▲) at a weight ratio of 100:1 (LDL protein:enzyme) for 6 h at 37°C. Small unilamellar vesicles were then added to obtain 2.00:1 incubation mixtures (SUV-PC:LDL-PL; LDL-PL, 0.554 mg/ml) and incubated at 37°C. Control LDL were also incubated with vesicles (■).
the data on the percent of LDL involved in aggregation. Of all incubation mixtures, the sample incubated at 5 °C had the highest amount of LDL involved in aggregation, 61%, and the highest final absorbance. The data also suggested that other factors may be involved. In particular, the absorbances for mixtures at 30 and 37 °C were similar despite the differences in the percent of LDL involved in aggregation -- 48% versus 55%, respectively. The percent of total LDL involved in aggregation would not be an appropriate measure of the aggregate concentration, if the aggregate molecular weight varied with temperature. Further studies, therefore, are necessary to obtain the molecular weight and radius of gyration for the aggregates in order to explain the molecular bases for temperature-dependent variations in absorbance.

N. ROLE OF APOLIPOPROTEIN B IN LDL-VESICLE INTERACTIONS

The role of apoB in the aggregation of LDL with vesicles was investigated by pre-treating LDL with either trypsin or papain at ratios of 100:1 (LDL protein:enzyme, wt:wt) for 6 h at 37 °C. These lipoproteins were then incubated (37 °C) with vesicles at a 2.00:1 weight ratio (SUV-PC:LDL-PL; LDL-PL, 0.554 mg/ml). Spectrophotometry (Fig. 33) of the mixtures and a control LDL-vesicle mixture showed that protease treatment significantly reduced the formation of light-scattering
Figure 34. Time course of change of absorbance of LDL incubation mixtures before and after trypsin addition. LDL (LDL-PL, 0.593 mg/ml) were incubated (37 °C) with vesicles at either a 2.00:1, o or a 4.00:1, o weight ratio (SUV-PC:LDL-PL) for 42 h. Trypsin was added to the mixtures at a 100:1 weight ratio (LDL protein:trypsin), and the absorbance was monitored (340 nm) for 6 h.
products. Pre-treatment of LDL with neuraminadase, however, did not affect the time course for the absorbance values (Fig. 33). The electrophoretic banding pattern for trypsin- or neuraminadase-treated LDL was similar to that for control LDL; papain-treated LDL, however, showed a slight decrease in subpopulation sizes (data not shown). These electrophoretic data suggested that enzyme-treated LDL had not undergone gross structural changes. Thus, apoB plays a role in the aggregation of LDL with vesicles, but the sialic acid component of this glycoprotein is probably not necessary for LDL-vesicle interaction.

The role of apoB in LDL-vesicle aggregation was also examined by investigating the effects of trypsin treatment on mixtures at 2.00:1 and 4.00:1 (SUV-PC:LDL-PL; LDL-PL, 0.600 mg/ml) weight ratios that had incubated (37 °C) for at least 40 h prior to enzyme addition. Trypsin was added to incubation mixtures at a weight ratio of 100:1 (LDL protein:trypsin); subsequently, the absorbances of the incubation mixtures were monitored for 6 h at 37 °C (Fig. 34). After trypsin addition, absorbances decreased rapidly (within 3 h) to limiting values that were about 35% of the absorbances just prior to trypsin addition. Gradient gel electrophoresis of the trypsin-treated mixtures showed an increase in staining intensities within the LDL migration range compared to the untreated samples (data not shown). In addition, trypsin treatment decreased the staining intensities near the top of the gel for the aggregation
products compared with the intensities in this migration range for untreated mixtures (data not shown). Some aggregation products, however, were still observed and probably were responsible for the residual absorbances noted for the incubation mixtures. These findings indicated that apoB was involved in the formation of aggregates and suggested that this apolipoprotein may link LDL with vesicles. Because of the residual absorbances observed for the incubation mixtures after trypsin treatment, the data also suggested that there were at least two types of protein linkages between LDL and vesicles -- one was trypsin-sensitive and the other was trypsin-resistant.

O. VESICLE PERTURBATIONS DUE TO LDL-VESICLE INTERACTIONS

Perturbation of the vesicle bilayer was monitored by following the release of entrapped carboxyfluorescein (377.3 MW) or $^3$H-inulin (about 5000 MW). Determination of the release of these molecules from vesicles is discussed in the Materials and Methods section. Possible mechanisms of vesicle perturbation during interaction with LDL include bilayer disruption during uptake of phospholipid by LDL and changes in bilayer integrity during aggregation.

Leakage of purified carboxyfluorescein (114 mM) from vesicles in the absence of LDL was investigated at
Figure 35. Time course of release of vesicle-entrapped molecules. Vesicles were incubated without LDL at 6, ○; 14, ▲; 30, ■; and 37 °C, ◆. The release of entrapped molecules from vesicles was monitored as described in the materials and methods section. A) Carboxyfluorescein release from vesicles. B) \(^{3}H\)-Inulin release from vesicles.
temperatures ranging between 6 and 37 C. Considerable leakage was observed at 30 and 37 C (Fig. 35A). Based on the initial rate (k) of leakage from vesicles, the activation energy for carboxyfluorescein diffusion through the vesicle bilayer was estimated. The calculated activation energy was approximately 35 Kcal/mole assuming a temperature independent coefficient (A) in the Arrhenius expression ($\ln k = \frac{E}{T} + \ln A$). This value is slightly larger than the activation energy of 24.6 Kcal/mole reported for the leakage of calcein from vesicles (210).

The trapped volume of the unilamellar vesicles was estimated using carboxyfluorescein. Based on an extinction coefficient of $58 \times 10^3$ (absorbance per mole) at 492 nm (244), the estimated average volume was 0.268 L/mole PC. This result agrees favorably with the minimum value of approximately 0.25 L/mole PC reported for unilamellar vesicles (247).

The behavior of vesicles containing $^3$H-inulin was also investigated. In the absence of LDL, incubations at 6, 14, 30, and 37 °C (Fig. 35B) showed no significant leakage of this molecule. Therefore, investigation of vesicle perturbation in the presence of LDL was performed using entrapped $^3$H-inulin.

Studies of LDL-vesicle interaction at a 1:1 weight ratio (SUV-PC:LDL-PL) were initially conducted at incubation temperatures of 6, 14, 30, and 37 °C. The results were corrected for inulin leakage from vesicles in the absence of LDL. Figure 36 clearly demonstrates that
Figure 36. Time course of release of inulin from LDL incubation mixtures. Vesicles containing entrapped \(^3\text{H}\)-inulin were incubated with LDL (LDL-PL, 0.593 mg/ml) at a 1.00:1 weight ratio (SUV-PC:LDL-PL). The release of inulin at incubation temperatures of 6, ○; 14, △; 30, ■; 37 °C, ◆ is shown after correction for the small amount of inulin release that occurs in the absence of LDL.
inulin release from vesicles is enhanced by the presence of LDL. At early times, inulin release appeared to be temperature dependent — release at 30 and 37 °C was more than that at 6 and 14 °C. At 48 h, however, there was no temperature dependence; inulin release approached a limiting value in the range of 55 to 60% of the maximum possible release. These data indicate that the vesicle bilayer is sufficiently perturbed in the presence of LDL to allow release of inulin. (Inulin is thought to be cylindrical in shape with a diameter and height of 2.0 and 5.0 nm, respectively (248, 249).)

P. LDL- Vesicle Interactions in Total Lipoprotein Mixtures

Since both HDL and LDL interact with vesicles, it was of interest to investigate the possible competition of these lipoproteins for vesicles. These studies specifically compared the extent of LDL aggregation for a mixture of total plasma lipoproteins (d<1.210 g/ml) versus that for the LDL isolated from the mixture. The extent of aggregation for LDL and the percent banding area for LDL are inversely related. (The percent banding area is the ratio of banding areas, expressed as a percentage, for LDL from mixtures incubated with and without vesicles.)

The lipoprotein samples were separately incubated for 24 h with increasing amounts of vesicles. The LDL (LDL-PL, 0.70 mg/ml) isolated from subject JH were incubated
Figure 37. Gradient gel electrophoresis of total lipoprotein incubation mixtures. Total (d<1.210 g/ml) lipoproteins from subjects JH and JB were incubated with various levels of vesicles. Isolated LDL (LDL-PL, 0.700 mg/ml) from subject JH were also incubated with vesicles. The densitometric banding area for LDL peaks in incubation mixtures are expressed as a percentage of the banding area for LDL incubated alone. Electrophoresis of the incubation mixtures was performed on 2-16% polyacrylamide gradient gels as described in the legend to Figure 9. The plasma lipoprotein levels of HDL and LDL for subject JH were 1.58 and 3.10 mg/ml, respectively. For subject JB, the concentrations were 3.23 and 1.90 mg/ml, respectively. Total lipoprotein incubation mixtures for subject JB (○). Total lipoprotein incubation mixtures for subject JH (●). LDL incubation mixtures for subject JH (▲).
(37 °C) for 24 h with vesicles at weight ratios of 0.50:1, 1.00:1, 2.00:1, and 4.00:1 (SUV-PC:LDL-PL). The total lipoprotein mixture from subject JH (LDL-PL, 0.70 mg/ml) was incubated with vesicles to give weight ratios of 0.66:1, 1.33:1, 2.66:1, and 5.65:1 (SUV-PC:LDL-PL). At comparable incubation ratios, Figure 37 shows the banding area for LDL to be less for the isolated lipoprotein class than for the total mixture of lipoproteins. Therefore, the extent of LDL aggregation was less for samples that contained HDL than for those that only had LDL. This finding suggested that HDL competed with LDL for vesicles. Indeed, densitometric scans showed an increase in peak diameters for HDL size subpopulations (data not shown). Similar findings were observed previously for isolated HDL density subclasses that had taken up phospholipid and lost apolipoprotein during incubations with vesicles (Figs. 6-8).

The extent of LDL aggregation was also compared for total lipoprotein mixtures that differed in the HDL to LDL weight ratios. For these studies, total plasma lipoproteins (d<1.210 g/ml) were isolated from two subjects (JH and JB). Based on analytical ultracentrifugation data, subject JH had respective LDL and HDL plasma concentrations of 3.10 and 1.58 mg/ml; less than 3% of the overall lipoprotein concentration was contributed by lipoprotein classes other than LDL and HDL. Subject JB had respective LDL and HDL plasma concentrations of 1.90 and 3.23 mg/ml;
in this case, less than 1% of the total lipoprotein concentration was due to lipoproteins other than LDL and HDL. The lipoprotein mixtures for each subject were separately incubated for 24 h with increasing amounts of vesicles.

The data for lipoprotein fractions (d<1.210 g/ml) from subjects JH and JB showed a linear decrease in the percent LDL banding area for weight ratio mixtures up to 3.0:1 and 6.0:1 (SUV-PC:LDL-PL), respectively (Fig. 37). At higher ratios, the percent LDL banding areas were less than expected by linear extrapolation of the data. Thus, for subject JH, the percent banding area for LDL at a 5.5:1 (SUV-PC:LDL-PL) weight ratio was 27% of the control value rather than 65%; for subject JB, the value at a 11:1 weight ratio was 50% rather than 79%. At comparable incubation ratios, the data also demonstrated a consistently higher percent LDL banding area for mixtures from subject JB than from JH. Thus, the extent of aggregation for LDL was greater in mixtures from JH than in mixtures from JB at comparable weight ratios (SUV-PC:LDL-PL). Since the respective HDL to LDL weight ratios for JH and JB were 0.51:1 and 1.69:1, the data suggested that the competition of HDL and LDL for vesicles depended on the relative amounts of these lipoproteins; higher HDL to LDL weight ratios resulted in a lower percent of LDL involved in aggregation.
IV. DISCUSSION

A. INTERACTIONS OF HDL WITH VESICLES

Incubation of each HDL density subclass (HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$) with small unilamellar vesicles of phosphatidylcholine resulted in the formation of three interaction products: HDL with an altered chemical composition; discoidal complexes of apolipoproteins, predominantly apoA-I, and phospholipid; and liposomes with associated apolipoproteins, predominantly apoA-I.

The altered composition of HDL resulted from an uptake of phospholipid by and a dissociation of apolipoprotein from the native lipoprotein species. The dissociated apolipoprotein was predominantly apoA-I, as previously reported by Hunter et al. (54) and by Tall and Green (55). Minor amounts of other apolipoproteins were also dissociated -- apoA-II from HDL$_3$ and HDL$_{2a}$ density subclasses and apoA-II and apoC from HDL$_{2b}$. For HDL$_3$ and HDL$_{2b}$, apolipoprotein dissociation was proportional to the molar ratio (PC:HDL) for respective values up to 295:1 and 1045:1. At these molar ratios, neither HDL$_3$ nor HDL$_{2b}$ was reaching a limit in apolipoprotein dissociation; the average number of apoA-I molecules dissociated per lipoprotein particle was estimated to be 0.8:1 and 2.0:1, respectively. At molar ratios up to 345:1
(PC:HDL), increasing amounts of apolipoprotein were
dissociated from HDL$_{2a}$. The amount of dissociated
apolipoprotein, however, approached a limiting value at
higher ratios. At a weight ratio of 710:1 (PC:HDL), the
average number of apoA-I molecules dissociated per HDL$_{2a}$
particle was 1.2:1.

The composition of HDL density subclasses was also
modified by phospholipid uptake. For HDL$_3$ and HDL$_{2a}$ at
respective molar ratios of 295:1 and 710:1 (PC:HDL), no
limit for phospholipid uptake had been reached. For
HDL$_{2b}$, however, phospholipid uptake approached a limit of
approximately 50 molecules per lipoprotein particle at
molar ratios greater than 535:1 (PC:HDL). When the data
were compared for incubation mixtures with molar ratios up
to 300:1 (PC:HDL), the different HDL density subclasses
could be ranked in terms of molar phospholipid uptake per
lipoprotein particle as follows: HDL$_{2a}$$>$HDL$_3$$>$HDL$_{2b}$.
At higher molar ratios, only HDL$_{2a}$ and HDL$_{2b}$ could be
compared; phospholipid uptake by HDL$_{2a}$ continued to be
greater than by HDL$_{2b}$.

Previous studies on the interaction of HDL$_3$ with
vesicles (6 h, 37 °C) demonstrated a 41% increase in
lipoprotein phospholipid (54), at a weight ratio of 2.5:1
(SUV-PC:HDL-PL). For mixtures incubated at 37 °C for 4
h, Tall and Green have reported that phospholipid uptake by
HDL$_3$ approached a limit of 0.25 mg of phospholipid per mg
of HDL protein at weight ratios between 1.0:1 and 1.5:1
(SUV-PC:HDL protein) (55). The present study (37 °C, 6 h) showed a 44% increase in the phospholipid content of HDL₃, in good agreement with previous findings of 41%, at a weight ratio of 2.5:1 (SUV-PC:HDL-PL). A value of 0.32 mg of phospholipid taken up per mg of HDL protein was obtained at a weight ratio between 1.0:1 and 1.5:1 (SUV-PC:HDL protein). The disparity between my value of 0.32:1 and that of 0.25:1 (mg of phospholipid uptake per mg of HDL protein) obtained by Tall and Green (55) may be accounted for, in part, by the difference in our incubation times -- 6 versus 4 h, respectively. A further distinction between the findings of our respective studies concerns the saturability of HDL₃ to phospholipid uptake. In contrast to the results of Tall and Green, at similar weight ratios, no saturation of HDL₃ by phospholipid was apparent in my data. A possible explanation for this difference may be that their isolation of the HDL₃ density subclass required a total ultracentrifugation time of 108 h, while isolation of the HDL₃ subclass in my studies required a total ultracentrifugation time of only 72 h. Since, as described in their report, transfer of PC radioactivity to HDL and, presumably, net transfer of PC mass was progressively reduced with increasing ultracentrifugation time, uptake in their incubation system would be less than in my interaction system.

Tall and Green also examined phospholipid uptake by density subfractions of total HDL at a single weight ratio
of 0.2 mg of vesicle phospholipid per 0.4 mg of HDL protein. Expressed as mg of phospholipid per mg of HDL protein, they found the greatest uptake of phospholipid by subfractions within the HDL3 density range (1.125-1.210 g/ml). When my phospholipid uptake data are expressed this way, my findings for HDL3, HDL2a, and HDL2b also showed the greatest uptake by HDL3. On a molar basis, however, my data show the greatest uptake of phospholipid by HDL2a, as previously noted (Fig. 19).

The lipoprotein diameter increased with uptake of phospholipid and dissociation of apolipoprotein. Mean diameters of HDL size subpopulations were examined at both 6 and 24 h of incubation. The findings for the 6 h incubations were used for examination of the influence of lipoprotein chemical composition on particle size. The mean diameter for the (HDL2b)gge size subpopulation remained relatively constant at molar ratios up to 290:1 (PC:HDL), despite changes in lipoprotein chemical composition. At higher molar ratios, the mean diameter progressively increased. For the (HDL2a)gge size subpopulation, the mean diameter increased with an increase in molar ratio but generally remained in the (HDL2a)gge size range. The (HDL3a)gge and (HDL3b)gge mean diameters were more responsive to increasing molar ratios than the mean diameter for either (HDL2a)gge or (HDL2b)gge. At a 150:1 molar ratio (PC:HDL), the (HDL3a)gge mean diameter entered the (HDL2a)gge size
range. The (HDL\textsubscript{3b})gge mean diameter entered the
(HDL\textsubscript{2a})gge size range at a ratio of 295:1. None of the
mean diameters approached a limiting value after a 6 h
incubation period.

After a 24 h incubation, the mean diameter for each
HDL size subpopulation increased compared to the diameter
for the corresponding subpopulation at 6 h. The
(HDL\textsubscript{2b})gge mean diameter did not increase from the
control value until molar ratios greater than 110:1
(PC:HDL) were examined. At molar ratios greater than 345:1
(PC:HDL), the (HDL\textsubscript{2a})gge mean diameter entered the
(HDL\textsubscript{2b})gge size range. The (HDL\textsubscript{3a})gge mean diameter
entered the (HDL\textsubscript{2a})gge size range at the lowest molar
ratio of added vesicles (40:1); in fact, at the highest
molar ratio (295:1, PC:HDL), (HDL\textsubscript{3a})gge entered the
(HDL\textsubscript{2b})gge size range. The (HDL\textsubscript{3b})gge mean diameter
entered the (HDL\textsubscript{2a})gge size range at the highest molar
ratios (150:1 and 295:1, PC:HDL). At the highest molar
ratios examined for HDL\textsubscript{3}, HDL\textsubscript{2a}, and HDL\textsubscript{2b} (295:1,
710:1, and 1045:1, respectively), the diameter for each HDL
size subpopulation approached a limiting value after a 24 h
incubation.

In addition to the formation of HDL with an altered
chemical composition and diameter, interaction of HDL
density subclasses with vesicles also produced discoidal
complexes of apolipoprotein and phospholipid. These
products accounted for more than 75% of the apolipoprotein
dissociated from HDL at all molar ratios examined. The apolipoprotein component of the discoidal products was predominantly apoA-I; trace amounts of apoA-II were also detected.

Several peaks in the GGE patterns of each incubation mixture were detected that corresponded to the discoidal products. The cumulative data for the apparent diameters of the discoidal products showed a bimodal frequency distribution over the range of observed diameters, 21.0-37.0 nm; the most frequently observed apparent diameters fell within the size intervals of 26.0-27.0 and 30.0-31.0 nm. Tall and Green also reported the formation of discoidal products during incubations of HDL₃ with vesicles (55). Based on electron microscopy, they reported diameters in the range of 16.2 to 40.6 nm with a mean ± SE of 22.4 ± 0.6 nm. Recently, it has been shown that diameters of discoidal complexes based on EM measurements are actually larger than those based on GGE data (using globular proteins for calibration of particle size) (250,251). Thus, the discoidal complexes observed in my studies were larger than those noted by Tall and Green (55).

Discoidal complexes of phospholipid and apolipoproteins A-I and A-II have been isolated from the plasma of LCAT deficient patients and had diameters ranging from 13 to 24 nm based on EM (251). Discoidal complexes produced from apoA-I and phosphatidylcholine have diameters
ranging from 10.0 to 20.0 nm when formed by sonication and from 12.0 to 16.0 nm when formed by cholate dialysis (252, 253). (These complexes also contained 20 molar percent unesterified cholesterol.) Recently, data reported by Nichols et al. on discoidal complexes of apoA-I and phosphatidylcholine, formed by cholate dialysis, showed the disc diameter to be positively correlated with the initial molar ratio of phosphatidylcholine to apoA-I (250). The diameters of the complexes, based on EM, ranged from 10.4 to 26.4 nm. Thus, the discoidal complexes observed in my studies are larger than those produced by in vitro recombination or those observed for LCAT deficient patients.

For incubations of HDL₃ and vesicles, Tall and Green previously reported a mean hydrated density of approximately 1.050 g/ml for the discoidal product (55). This value agrees well with the mean hydrated density of 1.051 g/ml, described in this dissertation, for the discoidal product of HDL₃ incubations.

The data of Tall and Green differ substantially from my data on the phospholipid to protein weight ratio for the discoidal product. In my studies on separate incubations of HDL₃, HDL₂ₐ, and HDL₂ₐ, the respective mean phospholipid to protein weight ratios for the discoidal products were 4.98:1, 6.52:1, and 4.06:1; these weight ratios correspond to molar ratios of 185:1, 240:1, and 150:1 (PC:apoA-I), respectively. The weight ratio for the
HDL$_3$ product reported by Tall and Green was 9:1. Recently, discoidal complexes, formed by cholate dialysis, had PC to apoA-I molar ratios of 163:1 and 189:1 for species designated class 6 and 8, respectively.

Finally, liposomal complexes of apolipoprotein and phospholipid were also formed during the incubation studies. These products accounted for less than 25% of the apolipoprotein dissociated from HDL at all incubation ratios. For the liposomal product isolated from interaction mixtures containing either HDL$_3$ or HDL$_{2a}$, the only detectable apolipoprotein component was apoA-I. For the product isolated from HDL$_{2b}$ incubation mixtures, however, the components were the A-I and C apolipoproteins. Previous studies also noted the association of apolipoprotein with vesicles during incubation of liposomes with HDL$_3$, but this product was not characterized (55).

In the present study, the phospholipid to protein weight ratio for the liposome products was in the range of 20:1 to 22:1 for HDL$_3$ and HDL$_{2a}$ but was in the range of 5:1 to 12:1 for HDL$_{2b}$.

Because all three incubation products contained phospholipid, it was of interest to examine the distribution, among the products, of phospholipid that was added to incubation mixtures. For most incubation ratios, HDL took up between 20 and 30% of the added phospholipid; on the other hand, the discoidal products generally accounted for between 60 and 75% of the added phospholipid.
The liposomal product appeared at high incubation ratios and, at most, accounted for about 30% of the added phospholipid. Thus, while previous studies have emphasized the uptake of phospholipid by HDL during incubation with vesicles, the present work shows that the phospholipid added to incubation mixtures goes predominantly into formation of discoidal products.

B. MODELS OF HDL-VESICLE INTERACTIONS

At least two models can be developed to describe formation of the products noted during HDL-vesicle incubations. First, interaction of HDL with vesicles may involve a transient collision complex wherein apolipoproteins, primarily apoA-I, transfer to vesicles while phospholipids are taken up by HDL. In this scheme, the apolipoprotein-vesicle complexes would ultimately rearrange to form the discoidal product. Indeed, previous studies have shown that the lipid component of discoidal complexes is predominantly obtained from vesicles (55). The liposomal product observed at high incubation ratios would result from apolipoprotein-vesicle complexes which do not rearrange to form the discoidal product. This may be due to high PC:apoA-I weight ratios in the liposomal product; weight ratios in the range of 4:1 to 6.5:1 are observed for discoidal complexes. The HDL product results from the transfer of phospholipid to HDL and the transfer
of apolipoproteins to vesicles.

Second, the interaction of HDL with vesicles may involve transfer of monomeric forms of both apoA-I and phospholipid through the aqueous milieu. In this scheme, lipid-free apolipoproteins, particularly apoA-I, would initially dissociate from HDL; then, the water-soluble molecules of apolipoprotein would have several possible fates: reassociation with HDL, formation of an apolipoprotein-vesicle complex, or aggregation to form oligomers of apoA-I (254). As noted in the first model, the discoidal product would be formed by a rearrangement of the apolipoprotein-vesicle complex; the liposomal product would be formed from apolipoprotein-vesicle complexes which could not rearrange to form the discoidal products. Net transfer of phospholipid from vesicles to HDL would involve water-soluble monomers of phospholipid that have dissociated from the vesicle. These molecules would have two fates: reassociation with vesicles or insertion into the surface monolayer of the lipoprotein particle. The latter fate, along with changes in apolipoprotein content, would give rise to the HDL product.

Previous investigations have demonstrated the occurrence of several of the conditions necessary for the aqueous-monomer model of HDL-vesicle interaction: (a) monomers of lipid-free apoA-I in equilibrium with native HDL (255), (b) monomers of phospholipid can be detected in equilibrium with vesicles (256), and (c) formation of
discoidal and liposomal complexes does occur from interaction of lipid-free apoA-I with PC vesicles (257-261). In addition, the presence of lysolecithin (262) or apoA-II (263) facilitates the interaction of apoA-I with phospholipids.

Additional studies will be required to characterize the mechanism of HDL-vesicle interaction in solution. It is of interest to note, however, that transfer of phospholipid from vesicles to vesicles (264), from vesicles to cells (265), and from recombinant complexes (apolipoprotein and phospholipid) to recombinant complexes appears to involve aqueous monomers of lipid rather than a collision-complex between reactants (266).

In both of the above models for HDL-vesicle interaction, the change in HDL apolipoprotein and phospholipid components can occur either simultaneously or sequentially. The GGE data suggested that lipoprotein composition changed by a sequential process. For HDL₃ and HDL₂a at molar ratios below 295:1 (PC:HDL), the average number of apoA-I molecules dissociated per HDL particle was less than 1:1. Consequently, some of the lipoprotein particles maintained their initial complement of apolipoproteins. If a simultaneous change of apolipoprotein and phospholipid was required, then gradient gel patterns would be expected to show two peaks -- one peak corresponding to HDL that had lost one apoA-I and taken up phospholipid and a second peak corresponding to
HDL that had not changed in composition. Instead of two peaks, the GGE patterns showed a shift of the entire densitometric profile of HDL towards shorter migration distances. The data for HDL\textsubscript{2b} also showed that a change in apolipoprotein content did not necessarily require a simultaneous change in phospholipid content. For incubation mixtures at molar ratios above 535:1 (PC:HDL), phospholipid uptake appeared to reach saturation, but apolipoprotein continued to dissociate with increasing molar ratio.

While apolipoprotein dissociation and phospholipid uptake were not necessarily simultaneous events, linear regression analyses indicated that these compositional changes were highly correlated over a wide range of molar ratios. Thus, 35-40 phospholipid molecules were taken up per apoA-I molecule dissociated from HDL\textsubscript{3} and HDL\textsubscript{2a}, as determined from the data in Figure 22 A,B. For HDL\textsubscript{2b} at molar ratios up to 290:1 (PC:HDL), the slope of the apolipoprotein and phospholipid data gave a molar ratio of 39:1 (phospholipid uptake:apoA-I dissociated) -- similar to linear regression data for HDL\textsubscript{3} and HDL\textsubscript{2a}. Beyond this molar ratio, phospholipid uptake by HDL\textsubscript{2b} approached a limiting ratio of about 50:1 (PL:HDL,mol:mol), but apolipoprotein continued to dissociate from the lipoprotein particle. Consequently, the overall linear regression data for HDL\textsubscript{2b} were different from those obtained for HDL\textsubscript{3} and HDL\textsubscript{2a} in both slope and correlation coefficient. It
remains to be determined whether apolipoprotein dissociation and phospholipid uptake would remain highly correlated when HDL₃ and HDL₂a are saturated with phospholipid.

Consideration of both models for HDL-vesicle interaction raises a number of interesting questions: What fraction of the vesicle phospholipid can a lipoprotein particle take up? How many discoidal products can be formed from a single vesicle? How does the initial apolipoprotein-vesicle complex rearrange to form the discoidal product? The data presented in this dissertation can address the first two questions. Assuming a phospholipid uptake of, at most, 70 molecules per HDL particle, uptake of phospholipid by a single HDL particle would decrease the vesicle phospholipid content by only 3%. Assuming a diameter for the discoidal product between 26.0 and 30.0 nm and estimating that a phospholipid molecule occupies an area between 0.5 and 0.7 nm², one discoidal product has approximately the same number of phospholipid molecules as one vesicle. The question concerning the formation of a discoidal product from an apolipoprotein-vesicle complex requires further investigation.
C. IMPLICATIONS OF HDL-VESICLE INTERACTION DATA FOR THE STRUCTURE OF HDL

The present studies on the interaction of vesicles with HDL provide insight into the structure of these lipoproteins. At molar ratios from 535:1 to 1045:1 (PC:HDL), apolipoprotein could dissociate from HDL\textsubscript{2b} without further uptake of phospholipid. Since the HDL\textsubscript{2b} diameter also increased over this range without an increase in components, it appeared that this phospholipid-saturated lipoprotein could passively increase in size when apolipoprotein was removed. This finding suggests that the particle size of phospholipid-saturated lipoproteins may be controlled by apolipoprotein-apolipoprotein interactions on the surface of the lipoprotein particle that may give rise to a constraining network. Apolipoproteins are, indeed, located on the lipoprotein surface and are accessible to the aqueous milieu (267). Grow and Fried proposed interactions between apoA-I and apoA-II based on their studies using cross-linking reagents (268).

For HDL\textsubscript{3} and HDL\textsubscript{2a} incubation mixtures at a range of molar ratios less than 295:1 (PC:HDL), the average number of apoA-I molecules dissociated per lipoprotein particle was less than 1:1. Densitometry of gradient gels demonstrated a shift, with increasing mole ratios, towards shorter migration distances of the entire profile. Thus, lipoproteins that did and did not lose an apolipoprotein
molecule increased in particle size. The increase in particle size of lipoproteins that did not lose an apolipoprotein must be due to phospholipid uptake alone. It appears, therefore, that lipoproteins, consisting of a core of apolar lipids and a surface monolayer of apolipoproteins and polar lipids, can increase in particle size by incorporation of phospholipids without a concomitant increase in core components. There are at least two possible structural responses of HDL to phospholipid uptake: (1) maintenance of an approximately spherical shape or (2) transformation to an ellipsoidal shape. The former response would result in a greater increase in particle volume than the latter. If a spherical particle structure is maintained, then the volume of the lipoprotein's core would increase. This increase in core volume may allow an increase in the range of motion for the apolar lipids. On the other hand, if the lipoprotein particle becomes ellipsoidal in shape during phospholipid uptake, then the volume occupied by the core components may not differ appreciably from that found for the native particle. Further studies will be necessary to elucidate the structural response of HDL to phospholipid uptake.

Although apolipoproteins appeared to constrain the particle's diameter when lipoproteins were saturated with phospholipid, they did not constrain the diameter when lipoproteins were not saturated -- the lipoprotein diameter
could increase by phospholipid uptake alone. Therefore, the role of apolipoproteins in affecting lipoprotein size may depend on the amounts and packing of other surface components, such as phospholipids.

Finally, because apoA-I was the predominant apolipoprotein component dissociated from HDL density subclasses, the incubation data suggested that apoA-I had a lower affinity for HDL than apoA-II. This conclusion is in accord with previous investigations on the relative affinities of apoA-I and apoA-II for HDL (269).
D. INCUBATION OF LDL AND OTHER APOB-CONTAINING LIPOPROTEINS WITH VESICLES

Incubation of LDL with small unilamellar vesicles resulted in the formation of two products: (1) LDL with a modified chemical composition and (2) aggregates of LDL with vesicles. The change in chemical composition reflected phospholipid uptake by the LDL; in contrast to HDL, there was no evidence of apolipoprotein dissociation from LDL. The LDL-vesicle aggregates had an overall dimension greater than about 50 nm and comprised particles (29 ± 5.7 nm, mean diameter ± SD) somewhat larger in size than either native LDL (26.1 ± 3.1) or vesicles (27.3 ± 3.3).

Other apoB-containing lipoproteins (VLDL, IDL, and lipoprotein(a)) also formed aggregates with vesicles. As was noted for LDL, these apoB-containing lipoproteins demonstrated GGE findings indicative of aggregation with vesicles. The staining intensity decreased, within the migration ranges for the native lipoproteins, with an increase in weight ratio (SUV-PC:lipoprotein phospholipid) for the interaction mixture; near the top of the gradient gel, a band progressively appeared (data not shown). For these lipoproteins, possible changes in their chemical composition were not determined.

Formation of LDL-vesicle aggregation products
considerably increased the solution turbidity. During incubation at 37 °C, the absorbances increased and approached limiting values at 24 h. These values were proportional to the initial weight ratios (SUV-PC:LDL-PL) of the incubation mixtures. Interactions at incubation temperatures of 5, 14, 24, 30, and 37 °C were investigated to obtain an activation energy for LDL-vesicle aggregation. At these temperatures, all incubation mixtures with an initial 2:1 weight ratio (SUV-PC:LDL-PL) reached limiting absorbances by 72 h; the final absorbance values varied with temperature. Mixtures incubated at 5 °C consistently exhibited final absorbances greater than those for mixtures at 14 °C, and the mixtures incubated at 14 °C had absorbance values greater than those incubated at either 24, 30, or 37 °C. These differences in limiting absorbance values may have resulted from a temperature-dependence of either aggregate concentration, aggregate stoichiometry, or both.

Velocity ultracentrifugation was used to isolate vesicles, LDL, and aggregation products from mixtures incubated (24 h) at 5, 14, 30, and 37 °C. Chemical characterization of the ultracentrifugal fractions showed that the amount of apoB in the aggregation product at 5, 14, 30, and 37 °C was 61%, 57%, 48%, and 55% of the total LDL apolipoprotein, respectively.

Non-aggregated LDL showed an increase in phospholipid content in a temperature-dependent manner; at 5, 14, 30,
and 37 °C, LDL phospholipid increased by 0, 4, 23, and 31%.

The phospholipid:protein weight ratios of aggregation products at 5, 14, 30, and 37 °C were 2.83:1, 3.43:1, 3.98:1, and 3.46:1, respectively. These values were 3-4 times the weight ratio for native LDL (1.00:1). Since phospholipid uptake by LDL in the aggregation product might contribute to the observed weight ratios, these values were corrected by use of two separate models before molar stoichiometries (LDL:SUV) were estimated. The first model assumed that phospholipid uptake by aggregated LDL was the same as that by non-aggregated LDL; that is, 0%, 4%, 23%, and 31% at 5, 14, 30, and 37 °C, respectively. In this case, the molar stoichiometries estimated for the aggregates formed at 5, 14, 30, and 37 °C were 2.2:1, 1.2:1, 1.5:1, and 1.9:1 (LDL:SUV), respectively. The aggregation product formed at 5 °C had the highest estimated stoichiometry of the aggregation products.

The second model assumed that the total amount of phospholipid taken up by LDL could be estimated from the product of the fraction of total inulin released (Table V) and the total vesicle mass. The phospholipid taken up by LDL in the aggregation product was the difference between the total phospholipid taken up by all LDL and the amount determined for non-aggregated LDL. Based on this model, the percent phospholipid increase for aggregated LDL at 5, 14, 30, and 37 °C was 118%, 140%, 159%, and 144%,
respectively; the respective estimated molar ratios (LDL:SUV) were 6.3:1, 4.0:1, 3.0:1, and 4.0:1. For both models, the largest estimated stoichiometry of LDL:SUV in the aggregation product was observed for the mixture incubated at 5 °C.

Further studies are required to determine whether the phospholipid increase for non-aggregated LDL, based on chemical composition data, is actually much smaller than the phospholipid uptake by aggregated LDL, as estimated by inulin release. It is possible that the percent phospholipid uptake based on inulin release may be an overestimate since some inulin may leak out of the vesicles by mechanisms other than phospholipid uptake by LDL. For example, the interaction of apoB with the vesicle may sufficiently perturb the bilayer to allow release of the inulin molecule. Additional studies, using larger entrapped molecules, will be necessary to investigate phospholipid uptake by aggregated LDL.

E. MECHANISM OF LDL-VESICLE INTERACTION

Reaction kinetics of incubation mixtures (37 °C) were examined by use of the initial slopes from the absorbance versus time data. These studies were performed over a range of incubation weight ratios (SUV-PC:LDL-PL) with one reactant (either LDL or vesicles) at a high, hence relatively constant, concentration and the other reactant
at different initial concentrations. Under these conditions, the reaction kinetics were first-order with respect to either LDL or vesicles. Therefore, the overall reaction kinetics were second-order. This finding was consistent with an initial 1:1 (LDL:SUV) molar stoichiometry for the aggregate. As described above, the final stoichiometry formed at 37 °C was between 2:1 and 4:1 (LDL:SUV). Thus, at least one additional lipoprotein particle was added to the initial 1:1 product. If, for example, the molar ratio at 37 °C was 2:1, the data would not rule out a molar stoichiometry for the aggregate of 4:2, 6:3, or some higher multiple of 2:1. A molecular weight for the aggregation product will be needed to obtain the actual molar ratios. It will also be of interest to determine whether additional LDL added to the initial 1:1 (LDL:SUV) aggregate interact with the LDL, vesicle, or both particles.

Incubations were performed at various temperatures (5, 14, 24, 30, and 37 °C) to estimate an activation energy for LDL-vesicle aggregation. For incubation mixtures at a 2:1 (SUV-PC:LDL-PL) weight ratio, the rate data did not yield a linear Arrhenius plot. Consequently, an activation energy could not be calculated. Instead of a linear plot, an extrapolation of the data for initial rate versus incubation temperature showed a minimum initial rate at 28 °C. Investigations of LDL from different subjects showed the extrapolated minimum to occur at a temperature in the
range of 21 to 28 °C.

Deviations of the initial rate data from a linear Arrhenius plot may be due to a temperature dependence of the initial aggregation stoichiometry, the aggregation rate, or both. A temperature-dependence of the initial aggregate stoichiometry is unlikely because it would require the simultaneous collision of 3 or more reactants -- an unlikely event based on statistical considerations. Changes in aggregation rate may occur, however, because of some alteration in LDL structure with temperature. The cholesteryl ester core of LDL undergoes a transition from a fluid to a smectic liquid-crystalline phase at a specific temperature. The transition temperature varies for different LDL samples and has a transition peak at a temperature in the range of 26.0-36.5 with a mean ± SD of 30.3 ± 2.3 °C. (270,271); the mean onset temperature for the transition was 17.3 ± 3.3 °C (range 11-23 °C). It is possible that this structural change in LDL may affect the rate of aggregation. Additional factors appear to be involved, however, because the rate continues to increase with a decrease in incubation temperature to 5 °C. If the phase transition of cholesteryl ester was the only factor involved, one would expect a change in the reaction kinetics to occur during the phase transition which could not be accounted for by a change in temperature alone. If the activation energy remained constant below the temperature range of the phase transition, there would be a
decrease in the reaction rate with a decrease in temperature. In fact, the data show an increase in rate with a decrease in temperature. Thus, the activation energy apparently decreases at temperatures below the cholesteryl ester phase transition. The structural changes of LDL which would account for these findings are not evident at the present time. Perhaps the apoB conformation is temperature dependent below the transition temperature. Additional studies will be necessary to investigate this hypothesis.

The possible role of apoB in aggregate formation was investigated by protease treatment of LDL or the aggregation products. After treatment of LDL with papain or trypsin, very little increase in absorbance was observed for mixtures at a 1.00:1 (SUV-PC:LDL-PL) weight ratio. When trypsin was added to mixtures at 2.00:1 and 4.00:1 (SUV-PC:LDL-PL) weight ratios after a 40 h incubation, a rapid decrease in the absorbance occurred; a new limiting absorbance was attained within 3 h, which was about 35% of the absorbance just prior to trypsin addition. Gradient gel electrophoresis showed that protease-treated LDL were similar in size to native LDL so that gross structural changes in LDL did not account for the inability of these lipoproteins to aggregate with vesicles. Therefore, these studies suggested that apoB plays an important role in the formation of LDL-vesicle interaction products probably by linking LDL with vesicles. Moreover, the experimental
findings suggested that there may be at least two types of apoB linkages between LDL and vesicles: trypsin-sensitive and trypsin-resistant.

Additional evidence for apoB involvement in aggregation was obtained from incubation studies of other apoB-containing plasma lipoproteins, VLDL, IDL, and lipoprotein(a), with vesicles. Electrophoresis of incubation mixtures of these lipoproteins demonstrated their aggregation with vesicles, evidenced by a decreased staining intensity of the native lipoprotein band and an increased staining intensity near the top of the gel, as noted previously for LDL. Of all the lipoprotein classes investigated, only HDL did not aggregate with vesicles; they are the only lipoproteins that do not contain apoB. Thus, the data suggest that apoB is important for lipoprotein-vesicle aggregation.

F. IMPLICATIONS OF LDL-VESICLE INTERACTION DATA FOR THE STRUCTURE OF LDL

The maximum observed increase in the phospholipid composition of non-aggregated LDL was 31%. Thus, the molecular organization of lipoproteins allows for an increase in surface components without an obligatory increase in core components. For HDL, the chemical composition of the surface monolayer could also change without compensatory alterations in core components. In
contrast to HDL, apolipoprotein (apoB) remained associated with LDL; and the particle size stayed constant during phospholipid uptake. These findings can be explained by either of two hypotheses, which are not mutually exclusive: (1) phospholipid packing on the LDL surface may change to accommodate additional molecules. (2) conformational changes of apoB occur to accommodate uptake of phospholipid. Further experiments are necessary to examine these possibilities.

The kinetic data gave a non-linear Arrhenius plot with a minimum extrapolated rate at 28 °C. A series of samples of LDL gave minimum rates at specific temperatures, which were in the range of 21-28 °C. Because this range agrees well with the temperature range for the cholesteryl ester phase transition of LDL, the state of these apolar lipids, located in the core of LDL, probably affects the rate of interaction with vesicles. Moreover, since apoB is important for aggregation these findings suggest the intriguing possibility that the phase of cholesteryl ester components in the core influences the conformation or exposure of apoB on the surface of LDL.

The effect of trypsin on LDL-vesicle aggregation suggests that apoB has the capability to form either trypsin-sensitive, trypsin-resistant, or both types of interactions with vesicles. Two apoB molecules are estimated for each LDL particle. Additional studies will be required to answer the following questions: Does each
apoB molecule have the capacity to interact in a trypsin-sensitive and a trypsin-resistant manner? Does the apoB molecule change conformation below the phase transition temperature for the cholesteryl ester components of LDL?

G. INTERACTION OF TOTAL LIPOPROTEIN MIXTURES WITH VESICLES

Incubations of vesicles with total lipoprotein mixtures (these mixtures were isolated in the ultracentrifugal d<1.210 g/ml fraction) demonstrated some of the reaction products observed in separate incubations of HDL and LDL: (1) HDL with an increased particle diameter and (2) LDL-vesicle aggregation products. For incubation mixtures with LDL to HDL weight ratios of 1.96:1 and 0.59:1, the extent of LDL aggregation at a particular incubation ratio (SUV-PC:LDL-PL, wt:wt) was lower for the mixture having a larger HDL:LDL ratio. Furthermore, the aggregation data showed that the extent of aggregation was greater for isolated LDL than for the total lipoprotein mixtures (d<1.210 g/ml) at comparable incubation ratios (SUV-PC:LDL-PL,wt:wt). Thus, the presence of HDL reduces the extent of LDL-vesicle aggregation. This is probably due to competition between HDL and LDL for vesicle phospholipid.
H. COMPARISON OF LDL AND HDL INTERACTIONS WITH VESICLES

The interaction of major lipoprotein classes with vesicles can be classified according to the presence or the absence of apoB on the lipoprotein particle. ApoB-containing lipoproteins, LDL, VLDL, IDL, and lipoprotein(a), formed stable lipoprotein-vesicle aggregates whose dimensions were greater than those for the native lipoproteins. Low density lipoproteins formed aggregation products with dimensions greater than about 50 nm. ApolipoproteinB was important for linking the lipoprotein particle with the vesicle. On the other hand, lipoproteins that did not contain apoB (HDL) were not involved in aggregate formation. Instead, they took up phospholipid from vesicles and donated apolipoprotein to form discoidal complexes. Thus, apolipoprotein affinity for the parent lipoprotein is probably a major determinant of the type of lipoprotein-vesicle interaction. ApolipoproteinB has a high affinity for lipoproteins; in fact, it is not readily soluble in lipid-free form (272). The predominant apolipoproteins of HDL, apoA-I and apoA-II, are less avidly bound than apoB to their respective parent lipoproteins and are more soluble in lipid-free form. Therefore, HDL apolipoproteins -- particularly apoA-I, which has a lower affinity for HDL than apoA-II (269) -- transfer to vesicles. In contrast, apoB remains attached to the lipoprotein when apoB-containing lipoproteins
interact with vesicles and, thus, forms a protein link between the lipoprotein and vesicle.

Both HDL and LDL took up phospholipid. Non-aggregated LDL did not increase in particle diameter with phospholipid uptake. For HDL, both phospholipid uptake and apolipoprotein dissociation occurred and resulted in an increase of the mean diameters for HDL size subpopulations. The difference in response of the particle diameters of LDL versus HDL to changes in lipoprotein composition cannot be explained by variations in the amount of phospholipid taken up. For the conditions investigated, the three density subclasses, HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$, had maximum phospholipid increases of 68%, 54%, and 23% respectively. All these density subclasses demonstrated an increase in diameter with an increase of phospholipid content. For the conditions investigated, non-aggregated LDL had a maximum phospholipid uptake of 31% but did not increase in particle size. Thus, it appears that LDL has a greater capacity than HDL for phospholipid uptake without a compensatory change in particle diameter.
I. PHYSIOLOGICAL IMPLICATIONS

The catabolism of triacylglycerol-rich lipoproteins (CM and VLDL) results in the formation of a variety of products; one of which is a vesicular product (50,51,187). Studies of in vivo CM catabolism (187) and in vitro VLDL lipolysis (50) have suggested that the vesicles are single bilayers with diameters in the range of 30.0-65.0 nm and 35.0-80.0 nm, respectively. For the studies of VLDL metabolism (50), the vesicles were isolated in the density range of 1.019-1.063 g/ml but were not observed in the 1.063-1.210 g/ml density fraction. In contrast, the vesicles produced in vivo during CM lipolysis were isolated in both density ranges (187). At the present time, the discrepancy between these studies cannot be explained because the chemical composition of the vesicles has not been determined. Compositional data will be of importance for evaluating the in vivo implications of my in vitro findings. The cholesterol content of the vesicular product will be of particular importance because a number of studies have reported that this lipid reduces bilayer disruption, compared with pure phospholipid vesicles, during interactions with lipoprotein or plasma components (203,204-206,209-211,218,219). It will also be of interest to characterize the products of in vitro interactions between plasma lipoproteins and cholesterol-containing
vesicles. At the present time, the studies reported in this dissertation suggest several possible modes of in vivo interaction between plasma lipoproteins and vesicular products.

First, HDL may act primarily as a source of apolipoprotein for the production of discoidal complexes with phospholipid and secondarily as a site for phospholipid uptake. The present studies performed on all major HDL subclasses consistently showed that phospholipid added to HDL mixtures was predominantly incorporated into discoidal complexes rather than into HDL. In the bloodstream, both phospholipid-enriched HDL and discoidal products may take up cellular cholesterol. Uptake of cholesterol from cells by HDL has been reported previously (169,170). Interaction of discoidal products containing cholesterol with the LCAT enzyme may produce spherical HDL-like particles, as previously noted during incubation of discoidal complexes from LCAT-deficient patients with this enzyme (279). Phospholipid-enriched HDL may also serve as LCAT substrates. Thus, both of these HDL interaction products may effect the removal of cellular cholesterol. After formation of cholesteryl esters by LCAT, protein factor(s) may facilitate the transfer of this apolar lipid to other lipoprotein classes. Ultimately, this apolar lipid may be removed from plasma via hepatic binding of the acceptor lipoproteins. A fraction of this hepatic cholesterol may be secreted into bile as
cholesterol and bile acids. Thus, in this scheme, HDL products may initiate the uptake of cellular cholesterol and effect the ultimate removal of tissue cholesterol. Aspects of this pathway for cholesterol removal from cells have been discussed previously (273).

Second, aggregates of LDL with liposomes may also form in vivo. The fate of these complexes may have atherogenic implications. If LDL-vesicle aggregates adhere to sub-endothelial components, as observed for non-aggregated LDL (274,280), then this event would result in a high local concentration of unesterified cholesterol and cholesteryl esters. Therefore, these aggregates may contribute to a nidus of cholesterol deposition whose ultimate atherogenic potential would depend on the ability of cells in the arterial wall to utilize the cholesterol and on the ability of vascular components, such as HDL, to remove the cholesterol from its site of deposition. If these aggregates play a role in atherogenesis, then a protective role of HDL in atherosclerosis would involve their competition with LDL for the liposomal phospholipid.

Perhaps the most significant implication of these LDL-vesicle aggregation studies is the possible relevance of the findings to LDL-cell interactions. If LDL associates with the phospholipid of cell surfaces in a manner similar to that observed for vesicles, then LDL may be taken up during endocytosis at concentrations above that in the bulk interstitial fluid. This hypothetical
apoB-mediated association of LDL to phospholipid surfaces of cell membranes may explain the observed low-affinity binding and uptake of LDL to a variety of cell types. Initially, bulk fluid endocytosis was proposed as an explanation for such LDL uptake by fibroblasts (87). Recently, however, studies of monocyte-derived macrophages have demonstrated that uptake of LDL, at concentrations that saturate the high-affinity receptors, cannot be accounted for simply by bulk-fluid endocytosis (275). Unlike high-affinity binding and uptake of LDL, low-affinity uptake may not be regulated by cells; consequently, they would experience an uncontrolled influx of unesterified cholesterol and cholesteryl esters carried by LDL. If the above mechanism is involved in atherogenesis, there must be processes that allow LDL to interact with cells of the arterial wall. Studies by Hoff et al. have shown that apoB is found in the normal intima and media (276). There is some evidence that apoB found in atherosclerotic plaques may still be associated with LDL (277). Because of the insolubility of apoB in lipid-free form, it is likely that the presence of this apolipoprotein in the intima and media of the arterial wall or as a component of plaques indicates that intact LDL was present at these locations at one time.

Low density lipoproteins could interact with cells of the arterial wall, if the endothelial integrity was compromised. The endothelium can be disrupted by a variety
of means (278): hemodynamic forces, viruses, and chemicals (homocystine and cholesterol). Once the arterial intima is damaged, aggregation of LDL with cells, mediated by apoB, may occur. The development of atherosclerotic pathology would depend on the balance of factors at the vascular site — the rate of both cholesterol deposition versus the rate of cholesterol utilization by cells and cholesterol removal by vascular constituents, such as HDL.
V. SUMMARY AND CONCLUSIONS

Interaction of all density subclasses of HDL (HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$) with vesicles resulted in the formation of three products: liposomes with associated apolipoproteins, discoidal complexes of phospholipids and apolipoproteins, and lipoproteins with a modified chemical composition. The composition of HDL was altered by incorporation of phospholipid and dissociation of apolipoprotein, predominantly apoA-I. The lipoprotein particle diameter increased with these changes in chemical composition.

After interaction with vesicles for 6 h, the mean diameter of the (HDL$_{3a}$)gge size subpopulation, observed for mixtures at molar ratios above 150:1 (PC:HDL), shifted into the (HDL$_{2a}$)gge subpopulation size range; mean diameters for both (HDL$_{2a}$)gge and (HDL$_{2b}$)gge subpopulations increased with increasing molar ratios (PC:HDL) but remained in their respective subpopulation size ranges. After interaction for 24 h at respective molar ratios above 40:1 and 150:1, the mean diameters for both (HDL$_{3a}$)gge and (HDL$_{3b}$)gge subpopulations shifted into the (HDL$_{2a}$)gge subpopulation size range. At molar ratios above 345:1, the mean diameter of the (HDL$_{2a}$)gge subpopulation shifted into the (HDL$_{2b}$)gge subpopulation size range. The mean diameter of the (HDL$_{2b}$)gge
subpopulation increased but remained in the (HDL$_{2b}$)gge size range.

The discoidal product predominantly contained apoA-I, although minor amounts of apoA-II were also detected electrophoretically. For incubation mixtures containing HDL$_3$, HDL$_{2a}$, and HDL$_{2b}$, the average phospholipid to protein weight ratio of the discoidal product was 4.98:1, 6.52:1, and 4.06:1, respectively. This product generally accounted for 60 to 75% of the phospholipid added to the interaction mixtures, while phospholipid uptake by the lipoprotein product only accounted for 20 to 30%; less than 30% of the added phospholipid remained in vesicle form.

All apoB-containing lipoproteins, LDL, IDL, VLDL, and lipoprotein(a), formed aggregates with vesicles. For LDL, two products were observed: LDL with an altered chemical composition and LDL-vesicle aggregates. The LDL-vesicle reaction kinetics suggested that the initial aggregation product had a 1:1 (LDL:SUV) molar stoichiometry. Chemical characterization of the isolated aggregate, after a 24 h incubation, indicated that at least one lipoprotein particle was subsequently associated with the initial 1:1 aggregation product.

The data for initial aggregation rates at various temperatures (5, 14, 24, 30, and 37 °C) gave a non-linear Arrhenius plot with an extrapolated minimum rate occurring, for different LDL samples, at a temperature in the range of 21-28 °C. For all LDL samples investigated, the final
solution absorbance, resulting from formation of aggregation products, was temperature-dependent; mixtures incubated at 5 °C had higher final values than mixtures at 14 °C, and mixtures at 14 °C had higher absorbances than mixtures incubated at either 24, 30, or 37 °C. Among different LDL samples, there were no consistent trends for the absorbances of mixtures incubated at 24, 30, and 37 °C.

Comparison of the extent of LDL aggregation with vesicles for mixtures containing isolated LDL versus those containing LDL and HDL demonstrated that HDL competed with LDL for vesicle phospholipid. Moreover, HDL-containing lipoprotein mixtures with higher HDL:LDL weight ratios showed a lower extent of LDL aggregation.

The present findings suggest that a lipoprotein product similar in diameter to HDL_{2a} may be formed in vivo during interaction of HDL_{3} with the vesicular products of CM or VLDL metabolism. The chemical composition of native HDL_{2a}, however, cannot be accounted for by phospholipid uptake by HDL_{3} alone (24). Further chemical modification of the phospholipid-enriched HDL_{3} would be required to produce HDL_{2a} species.

The lipoprotein and discoidal products, which are phospholipid-rich, could take up cellular cholesterol and, thereby, play an important role in controlling the cholesterol levels in cell membranes in vivo. The action of lecithin:cholesterol acyltransferase would convert the
cholesterol, transported by the lipoprotein and discoidal products, into cholesteryl ester; this form of cholesterol would not transfer to cells and would ultimately be removed by the liver via hepatic binding of lipoproteins. The net effect of such processes would result in cholesterol removal from cells. These considerations form the basis for the putative role of HDL as a negative risk factor in atherogenesis. In addition, phospholipid uptake and cholesterol esterification may contribute to the in vivo heterogeneity of HDL.

My in vitro findings suggest that the diameter of LDL is not affected by a 31% increase in phospholipid content. Thus, the observed in vivo polydispersity in particle diameter is probably not solely due to various degrees of phospholipid uptake by nascent LDL. Phospholipid uptake in addition to other changes in chemical composition may, however, contribute to the observed heterogeneity in particle size, as well as other physical-chemical properties.

Finally, my findings suggest a molecular mechanism for the role of LDL in atherogenesis. The observed aggregation of LDL with vesicles, mediated by apoB, may be one aspect of a more general interaction between LDL and phospholipid surfaces. If this process occurs between LDL and cell membranes in the arterial wall, as well as between LDL and vesicles, then this lipoprotein property may have important implications for cardiovascular disease.
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